Preparation of submicron particles for theranostic applications : imaging and therapy

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Résumé

L'objectif de cette étude était de préparer et de caractériser les particules submicroniques multifonctionnelles utilisables simultanément pour le diagnostic et le traitement de plusieurs maladies mortelles telles que le cancer. Pour ce faire, une étude systématique a été réalisée afin de comprendre les mécanismes impliqués et d'optimiser les paramètres du procédé de double émulsion-évaporation de solvant pour la préparation de ces particules. Pour l'imagerie in vitro, des nanoparticules polymériques fluorescentes (FluoSpheres®) ont été encapsulées dans une matrice polycaprolactone dégradable en utilisant le procédé de l'émulsion double-évaporation de solvant. Pour l'imagerie in-vivo, des nanoparticules d'or colloïdal ont été préparées et encapsulées via le même procédé et parfaitement caractérisées. Enfin, pour application theranostic, les nanoparticules d'or (comme agent de contraste) et un actif moléculaire (hydrophile Nefopam et hydrophobe benzoate de benzyle) ont été encapsulés simultanément dans des particules de polycaprolactone. Ces particules multifonctionnelles ont été caractérisées et évaluées in vitro comme model de pénétration cutané.

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PART I

GENERAL INTRODUCTION

The application of nanotechnology in biomedical field for therapy of various diseases has received substantial attention in recent years. It offers a unique approach against fatal diseases like cancer, CVS, HIV and diabetes, through early diagnosis, prediction, prevention and personalized therapy[1,2]. It plays a vital role in target-specific drug therapy and techniques for early diagnosis of tumor cells. Nanomedicine has potential for revolutionizing cancer therapy and diagnosis by developing new biocompatible nanocarrier systems for drug delivery purposes. These nanosystem have four unique properties unlike conventional therapeutics.

(i) It can be attached to targeting ligands, which have high affinity and specificity for target cells. (ii) It can themselves possess therapeutic or diagnostic properties and can be utilize to carry therapeutic agents. (iii) It can be designed for loading multiple therapeutic agents simultaneously. (iv) It can bypass traditional drug resistance mechanism.

Due to targeting strategies nanocarrier can achieve high drug concentration in tumor cells while minimizing toxicity in normal cells, so enhancing the therapeutic effects and reducing systemic toxicity[3,4]. Combining diagnosis and therapy in one process is referred as theranostic, the basic goal of theranostic is to target specific tissue selectively, to monitor the response to the treatment, to increase drug efficacy and safety, to increase therapeutic and diagnostic accuracy[5]. This strategy can enable us to make the treatment shorter, safer and more efficient for fetal disease like cancer, HIV, diabetes etc. Biocompatible theranostic particles for cancer therapy are under development, which would accelerate the therapy, reduce the drug toxicity and side effects. Presently, most of the research in theranostic has been focused on the management of cancer, since it is the major disease and leading cause of mortality.

Cancer, also known as malignant tumor is a term used for a group of almost 100 diseases. Its two main characteristics are uncontrolled growth of the cells in the human body and the ability of these cells to migrate from the original site and spread to distant sites of the body through blood or lymph, the process is termed as metastasis. The most common cancers are skin cancer, lung cancer, colon cancer, breast cancer (in women), and prostate cancer (in men). Cancer is a major cause of mortality worldwide. In United States, one out of every four deaths is from cancer. About 1.2 million Americans are diagnosed with cancer annually; more than 500,000 die of cancer annually[6]. Normally cells (the structural and functional unit of life) grow and divide in a controlled way as they are needed to keep the body healthy. However, sometime this orderly process goes wrong. When the genetic material of cell (DNA) become damaged or abnormal due to the environmental and genetic

factors; consequently, there is uncontrolled proliferation of cells. These cells form a mass of tissue called "tumor" or neoplasm (new growth). All tumors are not cancerous; tumors are of two types, benign and malignant. (i) Benign tumors aren't cancerous. It is slow growing, does not spread or invade surrounding tissue, and once it is removed, doesn't usually recur. (ii) Malignant tumors are cancerous. Cells in these tumors can invade nearby tissues and spread to other parts of the body. The spread of cancer from one part of the body to another is called metastasis. Some cancers do not form tumors. For example, leukemia is a cancer of the bone marrow and blood.

In spite of many advances in conventional cancer therapy such as chemotherapy and radiation[7], it is still facing many challenges e.g nonspecific systemic distribution of therapeutic agents, inadequate drug concentrations reaching the tumor site, unbearable cytotoxicity, poor therapeutic drug response monitoring and development of multiple drug resistance[8–10]. Therefore, there is need of innovative technologies development that could overcome these challenges and help to properly identify residual tumor cells, outlines of tumor margins and determine whether a tumor has been totally removed[11]. For effective cancer therapy, the key issue is to achieve the appropriate concentration of antitumor agent in cancerous tissue with minimal loss of activity in blood circulation, and after reaching the target site, drug should have the ability to destroying tumor cell selectively with minimum damage to normal cells[12,13]. The efficacy of the cancer's treatment and the degree of change in the patient's quality of life is directly related to the treatment's capability to target and to kill the tumor cells while affecting as few healthy cells as possible. With this idea, it is essential to fabricate a single agent that could contribute potentially in cancer prevention, detection and treatment. Various types of tools/carriers have been developed to the date including liposomes, polymeric micelles, dendrimers, carbon nanotubes, quantum dots and submicron particles.

Submicron-size colloidal particles are widely studied due to their numerous applications in oncology. Therapeutic and diagnostic agent of interest are encapsulated within their polymeric matrix or adsorbed onto the surface of the particle[14], and targeted to specific sites by surface modifications, to interact with the receptor expressed on tumor cells. By coating the particles with polysorbates, they can deliver the drug across the blood-brain barrier, enabling brain targeting after intravenous administration of drug. Similarly the efficiency of protein anticancer drug can be enhanced by encapsulation of active molecule in polymeric particles and targeting to specific sites. Moreover, sub-micron particle can be loaded with multiple types of drugs (both lipophilic and hydrophilic) for efficient treatment of tumors , can be loaded with multimodal imaging contrast

agents[15] and additionally, specific ligands can be attached to their surfaces in order to target surface-bound molecules on cancer cells. Typically, polymeric theranostic particle can be consisting of three main components, i.e. biomedical payload, polymeric carrier, and surface modifier. Biomedical "payloads" comprise imaging agents such as, organic dyes, quantum dot, optical contrast agents, MRI contrast agents, CT contrast agents, etc and therapeutic agents include anticancer drugs, DNA, proteins etc. while, carrier should provide physical support and protection to payload during its delivery to specific target. Modifiers are attached to the surface of carrier particle in order to enhance its circulation time, increased barrier penetration ability and to provide target specific binding abilities[16]. The submicron carriers can be targeted to cancerous cells by using various targeting strategies. The most commonly used approach is to identify a specific biomarker that is aberrantly expressed on the surface of cancer cells, and then to load its related binding vector onto carriers to achieve recognition and tumor binding. The unique size scale of the polymeric particles allows achievement of an enhanced-permeability-and-retention (EPR) effect in tumor cells targeting.

Nanoparticle-based imaging and therapy have been widely investigated, which have the ability to co-deliver therapeutic and imaging functions. It allows for imaging to be performed not only before and after, but also during a treatment regimen. Being non-invasive, imaging techniques are considered advantageous over repetitive biopsies of multiple tumor lesions in cancer patients. Numerous imaging techniques such as, computed X-ray tomography (CT), optical imaging, magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon-emission computed tomography (SPECT), and ultrasound have been used for diagnosis of disease including cancer and neurodegenerative diseases [5,17,18]. These techniques make the visualization of target tissues possible. Techniques such as MRI and optical imaging depend on contrast agent to visualize the organ of interest, which highlighting the differences between tissues and could augment the efficiency of imaging techniques[19,20]. Iron oxide nanoparticles (IONP), quantum dots, carbon nanotubes, fluorescent dyes, gold nanoparticles and silica nanoparticles, have been investigated in the imaging setting and are good candidate for building up nanoparticle-based theranostics. Gold nanoparticles (AuNPs) have also been used as a contrast agent in MRI and other imaging techniques. They possess many unique features such as surface plasmon resonance , bioconjugation, chemical stability and biocompatibility and have been studied in a variety of imaging field, including, computed tomography (CT), photoacoustics and surface-enhanced raman spectroscopy (SERS)[21,22]. Gold nanoparticles can be synthesized in various forms such as, spheres, cubes, rods, cages and wires with accurate quality

control and in large capacity. Their particle size and morphology is very important since, it influence the physical properties e.g surface plasmon absorption etc of AuNPs[23]. For example, 10 nm spherical Au NPs have maximum absorbance at about 520 nm with characteristic red color, while changing the sphere shape to rod-like, on the other hand, can push the absorption to the NIR region (650–900 nm).

The aim of this work is to prepare sub-micron particle for theranostic applications i.e for diagnosis and therapy. For this purpose, initially an optimized particle was prepared by using polycaprolactone (PCL) as polymer and polyvinyl alcohol as a stabilizer via double emulsion solvent evaporation technique using ultra turrax. All parameters affecting the colloidal property of the particles during the process were investigated and optimized. Additionally, the submicron particles were also prepared and optimized via double emulsion evaporation technique using power ultrasound. In the next step, PCL particles were loaded with fluorescent contrast agent in the guidance of the optimized parameters obtained from our previous study, and characterized in term of morphology, loading efficiency, size and distribution of fluorescent material in PCL particles. And finally gold nanoparticle were prepared to be used as contrast agent and loaded into PCL particles along with active drug simultaneously for in-vitro evaluation.

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PART II
BIBLIOGRAPHY

II.I. Applications of particles prepared from preformed polymers in drug delivery systems

General summary

Over the past decade, biodegradable and biocompatible polymers are widely used for biomedical applications. Various polymer carriers with different characteristics have been developed by different techniques for drug encapsulation but only biodegradable and biocompatible polymers are suitable for drug delivery system. Polymeric encapsulation of active drug permits enhancement of drug bioavailability, targeted delivery of drug, sustained release of drug and thus, minimizing the toxicity and side effects. These formulations can protect sensitive drug like proteins from degradation when administered via oral route. Thus, they enhance the drug efficiency, patient compliance and allow better management of disease. In this review, we report the commonly used polymers and techniques for micro and nano- encapsulation of active molecules, the principal of each technique, their operating conditions and application in drug delivery system.

The choice of technique and selection of suitable polymer is crucial step. It depends on the physicochemical properties of the drug to be encapsulated and the polymer to be used. Polymers can be either natural or synthetic. Generally, synthetic polymers have more advantages over natural ones by offering wide range of modifications in properties. The selection criteria of polymer for a carrier system depend upon their mechanical properties and degradation rate needed for a particular application. Among natural polymers, chitosan, cyclodextrins and dextran and its derivatives are frequently used. Chitosan is a nontoxic biodegradable polymer and can be digested by liposome or chitinases, which are present in human intestine and blood. It has mucoadhesive properties due to its positive charge that enable it to interact with negatively charged mucosal surface. Dextran can also be used for mucosal drug delivery, its nasal microspheres can release drug for extended period of time due to bioadhesive properties. Cyclodextrins are widely used in drug formulations with poor water solubility and poor stability. Synthetic derivatives of cyclodextrins (amphiphilic cyclodextrins) have been used recently for preparation of nanoparticles with high drug-loading capacity and targeting properties. In synthetic biodegradable polymers, the polyester-based like poly(lactic acid), poly (glycolic acid), poly(lactic co-glycolic acid) (PLGA) and polycaprolactone (PCL) are widely investigated for drug delivery applications. PLGA is approved by US FDA for drug delivery systems in humans. It is used to improve the controlled drug delivery formulations. The degradation time can vary from several months to several years depending on the weight and copolymer ratio. For example, lactide is more

hydrophobic than glycolic acid, so lactide-rich PLGA are more hydrophobic, absorb less water, and so degrade more slowly. PCL is a biodegradable polymer first identified in 1973. The surface hydrophobicity depends on the molecular weight of PCL, and undergoes slower biodegradation. Lipophilic drugs are generally distributed uniformly in the matrix while hydrophilic tends to remain on the surface of the PCL formulation in adsorbed state. Different techniques have been used for encapsulation of active drug via polymers. For example, nanoprecipitation, emulsion diffusion, double emulsion evaporation, and spray drying techniques.

Nanoprecipitation is a simple, fastest and reproducible method. It require two miscible phases i.e organic phase (good solvent for polymer) and aqueous phase (bad solvent for polymer), the organic phase containing dissolved polymer is slowly added to the aqueous phase under magnetic stirring, which leads displacement of organic solvent from organic solution, consequently results in polymeric suspension at the end. Commonly used solvent for nanoprecipitation are ethanol and acetone etc, while frequently studied polymers include PCL, PLA and PLGA. For emulsion diffusion method, three liquid phases are required: an organic phase, aqueous phase and dilution phase. The organic phase containing hydrophobic drug is homogenized with aqueous phase comprising stabilizer, and subsequent addition of large volume of dilution phase enables the diffusion of organic phase from the dispersed phase, hence results in polymeric suspension.

Double emulsion evaporation (DEE) technique can be used for encapsulation of both lipophilic and hydrophilic drugs. There are two types of DEE: water in oil in water emulsion (w/o/w) and oil in water in oil emulsion (o/w/o). In case of w/o/w, aqueous solution of hydrophilic drug is homogenized with organic phase containing polymer to form first emulsion (w/o). This step is followed by dispersion of first emulsion into second aqueous phase containing appropriate stabilize under high shear homogenization or sonication to form w/o/w emulsion. Subsequent evaporation of organic solvent from emulsion under ambient temperature or by rotary evaporator leads to formation of particulate carrier suspension. Spray drying is a very cost effective method. In which, polymer containing drug solution is atomized and sprayed into a drying chamber where droplets are dried by hot air, the subsequent precipitation of polymer leads to the encapsulation of drug. The evaporation of solvent occurs in a very short period of time so this technique can be utilized for encapsulation of heat-sensitive drug molecules.

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Review article:

PARTICLES FROM PREFORMED POLYMERS AS CARRIERS FOR DRUG DELIVERY

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ABSTRACT

Biodegradable and biocompatible polymers are widely used for the encapsulation of drug molecules. Various particulate carriers with different sizes and characteristics have been prepared by miscellaneous techniques. In this review, we reported the commonly used preformed polymer based techniques for the preparation of micro and nano-structured materials intended for drug encapsulation. A description of polymer-solvent interaction was provided. The most widely used polymers were reported and described and their related research studies were mentioned. Moreover, principles of each technique and its crucial operating conditions were described and discussed. Recent applications of all the reported techniques in drug delivery were also reviewed.

Keywords: Drug delivery, particles, polymer, encapsulation, carriers, operating conditions

INTRODUCTION

Particulate carriers have gained tremendous interest during the last decades which permitted to deliver many hydrophilic and hydrophobic molecules. Obtained particles present small size which facilitates their absorption. These drug delivery systems protect active pharmaceutical ingredients from degradation, enhance biopharmaceutical properties and could provide passive or active targeting or sustained delivery. Biomedical applications of the developed carriers are continuously growing (Ahmad, 2013; Soares, 2013; Miladi et al., 2013). Although, they present different physicochemical properties, the used polymers are mainly biocompatible and biodegradable. A multitude of techniques are used to obtain these particles. These methods differ by their principles and the nature of drug molecules that could be encapsulated. Some successfully marketed products led to an enlargement of the applications and the interest given by researchers to these drug delivery systems. Choice of the technique and operating conditions is crucial to obtain formulations bearing good properties for *in vitro* and *in vivo* applications. In this review, we will focus on polymeric particles and give a scope about the most used polymers. We will also describe the common preformed polymer based techniques used

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for the encapsulation of drug molecules. We will also review the major applications of the developed particles during the last years and their main properties.

1. POLYMER-SOLVENT INTERACTIONS

Many techniques that rely on preformed polymers have been used for the preparation of particulate carriers. Although these methods are quite different, they generally share a unique principle which is polymer precipitation. Precipitation of the polymer occurs either when a non solvent is added or after subsequent decrease of its solubility in a solvent. Many parameters could influence polymer solubility such as, solvent nature, pH, salinity and temperature of the dispersion medium. Solubility of polyelectrolytes in water, for example, is highly pH and salinity dependent (Gennes, 1979), while that of poly(alkyl acrylamide) and poly(alkyl methacrylamide), is mainly temperature dependent (Elaissari, 2002). In fact, nanoprecipitation and emulsion based techniques are based on the addition of a non solvent to the polymer which causes its precipitation. However, ionic gelation technique, for instance, in which generally a polyelectrolyte is used as polymer, is based on the addition of a salt or an oppositely charged polymer. This results in a change in the salinity of the medium and the appearance of electrostatic interactions and thus, leads to polymer precipitation. The thermodynamic behavior of the polymer in a given solution is highly dependent on the Flory χ-parameter. This parameter is defined as the free energy change per solvent molecule (in k_BT units) when a solvent-solvent contact is shifted to a solvent-polymer contact. It is expressed by the following mathematical equations:

$$\chi = \frac{\Delta G}{k_B T} = \frac{\Delta H - T\Delta S}{k_B T} = \frac{1}{2} - A\left(1 - \frac{\theta}{T}\right)$$

Equation (1)

where kB and T are Boltzmann constant and temperature, respectively; A and θ parameters are defined as follows:

$$A = \frac{2\Delta S + k_B}{2k_B}$$ Equation (2)

$$\theta = \frac{2\Delta H}{2\Delta S + k_B}$$ Equation (3)

It can be seen that the A parameter is directly related to entropy changes, whereas θ temperature is a function of both entropic and enthalpic variations. When θ temperature = T, the corresponding Flory χ-parameter = 1/2, at which the second Virial coefficient is equal zero (Elias, 2003). The latter can be easily determined from light scattering measurements of a diluted polymer solution. At θ temperature conditions, the binary interactions among constituents will be negligible and only the excluded volume effects will be predominant. Consequently, the solvent will be a good solvent for the polymer when $\chi < 1/2$ and a poor one when $\chi > 1/2$ (Minost et al., 2012).

2. COMMONLY USED POLYMERS FOR ENCAPSULATION

Several polymers have been used for drug encapsulation but only biodegradable and biocompatible ones are suitable for biomedical applications. The biodegradability of a polymer is acquired by the presence of a labile function such as ester, orthoester, anhydride, carbonate, amide, urea or urethane in their backbone. These polymers could be of natural (polysaccharides and protein based polymers) or synthetic (polyesters) nature (Pillai and Panchagnula, 2001). The most commonly used polymers for drug encapsulation are polyesters (lactide and glycolide copolymers, poly-ε-caprolactone), acrylic polymers (polymethacrylates) and polyamides (gelatin and albumin). The selection of the right polymer is a crucial step to obtain particles that are suitable for a well-defined application. In fact, polymers' structures are highly differ-

ent and their surface and bulk properties are highly relevant for the obtaining of the desirable biological application. Copolymers could be also used to monitor the hydrophobicity of the materials. Some polymers are poly(ethyleneglycol) (PEG) copolymerized in order to decrease nanoparticle recognition by the reticular endothelial system. Table 1 contains examples of the most used biocompatible and biodegradable polymers in encapsulation. Some polymers, especially those having mucoadhesive properties, could also be used for coating the nanocarriers (Mazzaferro et al., 2012; Zandanel and Vauthier, 2012).

Table 1: Commonly used polymers

Materials	References
Polymers	
Natural polymers	
Chitosan	Elmizadeh et al., 2013; Fàbregas et al., 2013; Khalil et al., 2012; Konecsni et al., 2012; Du et al., 2009; Bernkop-Schnürch et al., 2006; Gan et al., 2005; Asada et al., 2004
Dextran	Liang et al., 2013; Dai et al., 2012; Sajadi Tabassi et al., 2008; Koten et al., 2003
Dextran derivatives	Kanthamneni et al., 2012; Kauffman et al., 2012; Aumelas et al., 2007; Miyazaki et al., 2006
Cyclodextrins	Çirpanli et al., 2009; Memişoğlu et al., 2003; Pariot et al., 2002; Lemos-Senna et al., 1998
Gelatin	Nahar et al., 2008; Balthasar et al., 2005; Vandervoort and Ludwig, 2004; Bruschi et al., 2003
Synthetic polymers	
Biodegradable polyesters	
PLGA	Gyulai et al., 2013; Beck-Broichsitter et al., 2012; Morales-Cruz et al., 2012; Beck-Broichsitter et al., 2011; Nehilla et al., 2008; Song et al., 2008; Budhian et al., 2007; Bozkir and Saka, 2005; Fonseca et al., 2002;Yang et al., 1999; Govender et al., 1999
PLA	Bazylińska et al., 2013; Fredriksen and Grip 2012; Kadam et al., 2012; Kumari et al., 2011; Ataman-Önal et al., 2006; Lamalle-Bernard et al., 2006; Hyvönen et al., 2005; Katare et al., 2005; Chorny et al., 2002; Leo et al., 2000
PCL	Behera and Swain, 2012; Guerreiro et al., 2012; Hernán Pérez de la Ossa et al., 2012; Khayata et al., 2012; Arias et al., 2010; Wang et al., 2008; Limayem Blouza et al., 2006; Tewa-Tagne et al., 2006; Yang et al., 2006; Le Ray et al., 2003; Chawla and Amiji 2002; Raval et al., 2011; Hombreiro Pérez et al., 2000; Benoit et al., 1999; Masson et al., 1997
Poly(lactide-co-glycolide-co-caprolactone)	Zhang et al., 2006
Acrylic polymers	
Eudragit	Hao et al., 2013; Das et al., 2010; Eidi et al., 2010; Trapani et al., 2007; Galindo-Rodríguez et al., 2005; Haznedar and Dortunç 2004; Pignatello et al., 2002
Others	
Polyvinylbenzoate	Labruère et al., 2010
Pegylated polymers	
Chitosan-PEG	Seo et al., 2009
MPEG-PCL	Falamarzian and Lavasanifar, 2010; Xin et al., 2010
PCL-PEG-PCL	Suksiriworapong et al., 2012; Huang et al., 2010; Gou et al., 2009
Poly(caprolactone)-poly(ethylene oxide)-polylactide	Hu et al., 2003
PLA-PEG	Sacchetin et al., 2013; Essa et al., 2010; Ishihara et al., 2010; Vila et al., 2005; Vila et al., 2004; Govender et al., 2000; Huang et al., 1997
PLA-PEG-PLA	Chen et al., 2011; Ruan and Feng 2003
MPEG-PLA	Zheng et al., 2010; Dong and Feng, 2007; Dong and Feng, 2004

2.1 Natural polymers

2.1.1 Chitosan

Chitosan is obtained by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and cell walls of fungi. It is a cationic and biodegradable polysaccharide consisting of repeating D-glucosamine and N-acetyl-D-glucosamine units, linked via (1-4) glycosidic bonds. Chitosan is non toxic and can be digested in the physiological environment, either by lysozymes or by chitinases, which are present in the human intestine and in the blood. These properties led to increased interest for this polymer in pharmaceutical research and industry as a carrier for drug delivery (Mao et al., 2010). In addition, chitosan has mucoadhesive properties owing to its positive charge that allows interaction with the negatively-charged mucosal surface. Consequently, the use of chitosan as a matrix (Patil and Sawant, 2011) or as a coating material (Mazzarino et al., 2012) in drug encapsulation had become a promising strategy to prolong the residence time, to increase the absorption of active molecules through the mucosa (Mao et al., 2010; Alpar et al., 2005) and also for targeted delivery (Park et al., 2010).

2.1.2 Dextran and its derivatives

Dextran polymers are produced by bacteria from sucrose. Chemical synthesis is also possible. These glucose polymers consist predominantly of linear α-1,6-glucosidic linkage with some degree of branching via 1,3-linkage. Dextran-based microspheres have got much attention because of their low toxicity, good biocompatibility and biodegradability, which are of interest for application in biomedical and pharmaceutical fields (Mehvar, 2000). Many detxran polymers such as Sephadex® (cross-linked dextran microspheres) as well as Spherex® (cross-linked starch microspheres) were used as carriers for drug delivery. Other derivatives of dextran and starch including diethyl aminoethyl dextran and polyacryl starch have also been used for mucosal drug delivery. Illum et al. (2001) proposed some mechanisms to explain absorption enhancement effects of cross-linked starch and dextran microspheres intended to nasal delivery which are: (1) Deposition of the microspheres in the less or non ciliated anterior part of the nasal cavity and slower nasal clearance; (2) Retention of the formulation in the nasal cavity for an extended time period because of the bioadhesive properties of the microspheres and (3) The local high drug concentration provided by the gelled system in close contact with the epithelial absorptive surface (Illum et al., 2001).

2.1.3 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides that contain at least six D-(+) glucopyranose units which are attached by α-(1,4) glucosidic bonds. They have been widely used for the formulation of drugs with bioavailability concerns resulting from poor solubility, poor stability and severe side effects. There are 3 natural CDs which are α-, β-, and γ-CDs (with 6, 7, or 8 glucose units respectively) (Challa et al., 2005). In addition, amphiphilic cyclodextrins are synthetic derivatives of natural cyclodextrins. Such derivatives are able to self-organize in water to form micelles and nano-aggregates, which is interesting for pharmaceutical applications, mainly, encapsulation (Gèze et al., 2002). In fact, amphiphilic cyclodextrins have recently been used to prepare nanoparticles and nanocapsules without surfactants and have shown high drug-loading capacity with favorable release properties (Lemos-Senna et al., 1998; Çirpanli et al., 2009; Duchêne, 1999). They have also been used for targeting and for increasing drug loading (Duchêne et al., 1999).

2.1.4 Gelatin

Gelatin is a natural polymer that is derived from collagen. It is commonly used for pharmaceutical and medical applications because of its biodegradability and biocompatibility in physiological environments. Gelatin is attractive for use in controlled release due to its nontoxic, bioactive properties and inexpensive price. It is also a polyampholyte having both cationic and anionic groups along with hydrophilic groups. Mechanical properties, swelling behavior and thermal properties of gelatin depend significantly on its crosslinking degree (Young et al., 2005).

2.2 Biodegradable polyesters

Polyester-based polymers are among of the most widely investigated materials for drug delivery. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers poly(lactic acid-co-glycolic acid) (PLGA) along with poly-ε-caprolactone are some of the well-defined biomaterials with regard to design and performance for drug-delivery applications.

2.2.1 PLGA

PLGA, a copolymer of lactic acid and glycolic acid, has generated tremendous interest due to its excellent biocompatibility, biodegradability, and mechanical strength. PLGA is approved by the US FDA and European Medicine Agency (EMA) in various drug delivery systems in humans. In order to improve the formulation of controlled drug delivery systems, an understanding of the physical, chemical, and biological properties of polymers is helpful. In fact, the polymer is commercially available with different molecular weights and copolymer compositions. The degradation time can vary from several months to several years, depending on the molecular weight and copolymer ratio (Danhier et al., 2012). For example, lactic acid is more hydrophobic than glycolic acid and, therefore, lactide-rich PLGA copolymers are less hydrophilic, absorb less water, and subsequently, degrade more slowly (Dinarvand et al., 2011). PLGA particles are widely used to encapsulate active molecules with a broad spectrum of pharmaceutical applications (Danhier et al., 2012; Menei et al., 2005; Singh et al., 2004).

2.2.2 PLA

PLA is a biocompatible and biodegradable synthetic polyester which undergoes scission in the body to monomeric units of lactic acid. The latter is a natural intermediate in carbohydrate metabolism. PLA possess good mechanical properties and it is largely used for the preparation of particles (Gupta and Kumar, 2007).

2.2.3 PCL

It was in 1930s that the ring-opening polymerization of PCL was studied. The biodegradable property of this synthetic polymer was first identified in 1973. PCL is suitable for controlled drug delivery due to its high permeability to many drugs and non-toxicity (Sinha et al., 2004). Molecular weight dependent surface hydrophobicity and crystallinity of PCL are the causes for its slower biodegradation in two distinct phases such as random non-enzymatic cleavage and enzymatic fragmentation. Lipophilic drugs are generally distributed uniformly in the matrix while hydrophilic drugs tend to move towards the interface and remain on the surface of PCL formulation in adsorbed state. Diffusion was described as the only possible mechanism by which the lipophilic drugs release from PCL particles as they were shown to be intact for a much longer duration *in vivo*. However, two phenomena could be implicated in hydrophilic drugs' release. Highly lipophilic drugs that resist complete diffusion are released upon surface erosion by enzymatic action while hydrophilic drugs that accumulate at the interface during the formulation processes are released by desorption at the initial period of release study

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or dosage intake. This results in a biphasic drug release pattern for PCL particles with much higher burst release for hydrophilic drugs than lipophilic ones (Dash and Konkimalla, 2012).

2.3 Pegylated polymers

Many of the above cited polymers could be conjugated to PEG chains, which allows the enhancement of their hydrophilicity and permits the obtaining of a stealth surface that could protect the prepared carriers from degradation by the cells belonging to the reticuloendothelial system. Conjugation to PEG confers also bioadhesive properties for the carriers (Yoncheva et al. 2005).

3. Used methods for the encapsulation of active molecules

3.1 Nanoprecipitation

The nanoprecipitation technique was first developed by Fessi et al. in 1986 (Devissaguet et al., 1991). The technique allows the obtaining of either nanospheres or nanocapsules. The organic phase could be added to the aqueous phase under magnetic stirring. This one-step process allows the instantaneous and reproducible formation of monodisperse nanoparticles. Nanoprecipitation is simple, is by far the fastest, most reproducible, and industrially feasible preparation procedure of nanospheres (Vauthier and Bouchemal, 2009). Practically, two miscible phases are required: an organic solvent in which the polymer is dissolved and an aqueous phase (non-solvent of the polymer). The most common used organic solvents are ethanol and acetone. Such solvents are miscible in water and easy to remove by evaporation. Some oils could be added to these solvents to allow the dissolving of the active (Rosset et al., 2012). As Figure 1 shows, the method is based on the addition of one phase to the other under moderate magnetic stirring which causes the interfacial deposition of a polymer after displacement of the organic solvent from the organic solution. This leads to the formation of a suspension of nanoparticles. The organic phase could be a mixture of solvents such as, mixture of acetone with water or ethanol etc. Similarly, the aqueous phase could consist of a mixture of non-solvents and could contain surfactants. Commonly used polymers are biodegradable polyesters, especially PCL, PLA and PLGA (Rao and Geckeler, 2011). Particle formation process includes three basic steps which are, particle nucleation, molecular growth and aggregation. The rate of every step has a crucial impact on the particle size distribution. Supersaturation is the driving force that manages all of these steps, namely, particles nucleation rate. Supersaturation, itself, is influenced by fluid dynamics and mixing. In fact, low stirring rate results in low nucleation rates while higher mixing rates give high nucleation rates (Lince et al., 2008).

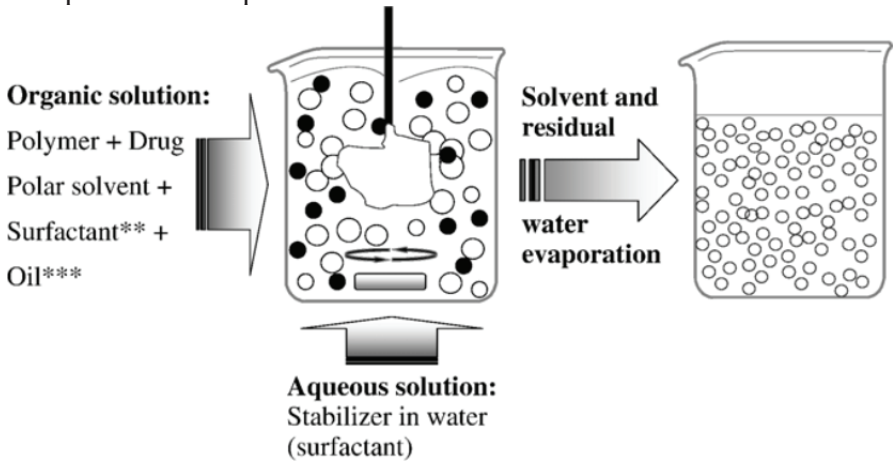

Figure 1: The nanoprecipitation technique (Pinto Reis et al., 2006)

Operational parameters that should be controlled include the organic phase to non organic phase ratio, the concentration of the polymer and the stabilizer and the amount of the drug. Every one of these parameters may exert an impact on the characteristics of the obtained nanoparticles (size, uniformity and charge). In fact, an increase of the polymer amount generally increases particles' size (Chorny et al., 2002; Simşek et al., 2013; Dong and Feng, 2004; Asadi et al., 2011). The same effect was obtained after increasing the polymer molecular weight (Limayem Blouza et al., 2006; Holgado et al., 2012). These findings were explained by an increase of the viscosity of the organic phase which rendered solvent diffusion more difficult and thus, led to larger nanoparticles' size. The effect of increasing organic phase volume seems conflicting: some studies showed that it causes a decrease of the particles size (Dong and Feng, 2004) while others showed the opposite phenomenon (Asadi et al., 2011). Increasing the water phase amount leads to a decrease of the particles size as a result of the increased diffusion of the water-miscible solvent in the aqueous phase and thus, the more rapid precipitation of the polymer and formation of nanoparticles (Budhian et al., 2007). An increase of the surfactant amount generally causes a decrease of the particles size and reduces size distribution (Contado et al., 2013; Siqueira-Moura et al., 2013). Some studies did not, however, found significant change following surfactant amount increase (Dong and Feng, 2004). The nature of the surfactant may also influence the particles' size (Limayem Blouza et al., 2006). Increasing mixing rate decreases the particles size as it causes faster diffusion rate (Asadi et al., 2011). Theoretical drug loading may also influence particles size and drug loading (Govender et al., 1999). Nanoprecipitation is generally designed for the encapsulation of hydrophobic drug molecules (Seju et al., 2011; Katara and Majumdar, 2013; Seremeta et al., 2013). Such actives may be dissolved within the organic phase. Bilalti et al. (2005) described a nanoprecipitation technique intended to the encapsulation of hydrophilic molecules but the size of the obtained particles was not sufficiently uniform (Bilati et al., 2005). To further improve the reproducibility of the nanoprecipitation technique and make it more convenient for industrial applications, membrane contactor and microfluidic technology were successfully used (Khayata et al., 2012; Xie and Smith, 2010). These techniques allow better size control within different batches of particles. Table 2 contains some examples of the applications of the nanoprecipitation technique in drug delivery during the last years. It can be concluded that polyesters are among the most used polymers for the preparation of the nanoparticles by this technique.

3.2 Emulsion diffusion (ESD)

ESD was first developed by Quintanar-Guerrero and Fessi (Quintanar-Guerrero et al., 1996) to prepare PLA based nanospheres. Three liquid phases are needed in this technique: an organic phase, an aqueous phase and a dilution phase. The organic phase generally contains the polymer and the hydrophobic drug. The aqueous phase is a solution of a stabilizing agent while the dilution phase usually consists of a large volume of water. Mutual saturation of the aqueous and organic phase allows further obtaining of a thermodynamically equilibrated emulsion upon high speed homogenization. Subsequent addition of an excess of water enables the diffusion of the organic solvent from the dispersed phase resulting in precipitation of the polymer and the formation of the particles (Figure 2). Commonly used polymers in this method include PCL, PLA and Eudragit® (Mora-Huertas et al., 2010). Table 3 shows that the technique is mainly used for the encapsulation of hydrophobic molecules. However, hydrophilic molecules may also be encapsulated by a modified solvent diffusion method using an aqueous inner phase (Ma

et al., 2001). Operating conditions affecting the size of the obtained particles include external/internal phase ratio, emulsification stirring rate, volume and temperature of water for dilution, polymer amount and concentration of the stabilizer (Quintanar-Guerrero et al., 1996; Mora-Huertas et al., 2010). Influence of high shear homogenization and sonication on the particles size was assessed and it was found that sonication was more efficient for particle size reduction. The nature of the surfactant influenced also the particles size. In fact, when Pluronic F68 (PF68), didodecyldimethylammonium bromide (DMAB) and polyvinylalcohol (PVA) were compared, DMAB gave the smallest particles but with the lowest encapsulation efficiency (Jain et al., 2011). Particles size was also described to increase with an increase of initial drug amount (Youm et al., 2012), polymer amount (Youm et al., 2012; Esmaeili et al., 2011) and the oil phase volume (Esmaeili et al., 2011; Poletto et al., 2008). An increase of the surfactant amount resulted in a decrease of the size but it seems that above some level further significant size reduction is no longer possible (Jain et al., 2011; Surassmo et al., 2010). An increase of the homogenization rate led to a decrease of the particles' size (Jain et al., 2011; Kwon et al., 2001; Galindo-Rodríguez et al., 2005). Likely, the same effect was obtained following an increase of the temperature and the volume of added water (Kwon et al., 2001; Song et al., 2006). The nature of the organic solvent also influenced particle size (Song et al., 2006). Table 3 shows some of the recent applications of the ESD technique.

Table 2: Applications of the nanoprecipitation technique

Encapsulated molecule	Polymer	Size (nm)	Zeta potential (mV)	Reference
Doxorubicin	Gelatin-co-PLA-DPPE	131.5-161.1	-	Han et al., 2013
Aceclofenac	Eudragit RL 100	75.5-184.4	22.5 - 32.6	Katara and Majumdar, 2013
Doxorubicin	Dextran-b-polycaprolactone	95-123.3	-	Li et al., 2013
Chloroaluminum phthalocyanine	PLGA	220.3-326.3	-17.7-(-40.9)	Siqueira-Moura et al., 2013
Efavirenz	PCL and Eudragit® RS 100	89.5 - 173.9	-17.9-53.8	Seremeta et al., 2013
Paclitaxel	PLGA	50 - 150	-15 - (-20)	Wang et al., 2013
Retinoic acid	PLA	153.6-229.8	-27.4-(-20.9)	Almouazen et al., 2012
Brimonidine Tartrate	Eudragit® RL 100	123.5 - 140.2	13.1- 20.8	Khan et al., 2012
Vitamin E	PCL	123-320	-24.5-(-1.46)	Khayata et al., 2012
Paclitaxel	Hydrophobized pullulan	127.6-253		Lee et al., 2012
Curcumin	PCL, chitosan	104-125	(-0.099)-79.8	Mazzarino et al., 2012
Diclofenac	PCL	152	-50	Mora-Huertas et al., 2012
Amphotericin B	PLGA	86-153	-31.4-(-9.1)	Van de Ven et al., 2012
Epirubicin	Poly(butyl cyanoacrylate)	217-235	-4.5-(-0.1)	Yordanov 2012
Camptothecin	Beta-cyclodextrin PLGA PCL	281 187 274	-13 -0.06 -19	Cırpanlı et al., 2011
Naringenin	Eudragit® E	90	-	Krishnakumar et al., 2011
Olanzapine	PLGA	91.2	-23.7	Seju et al., 2011

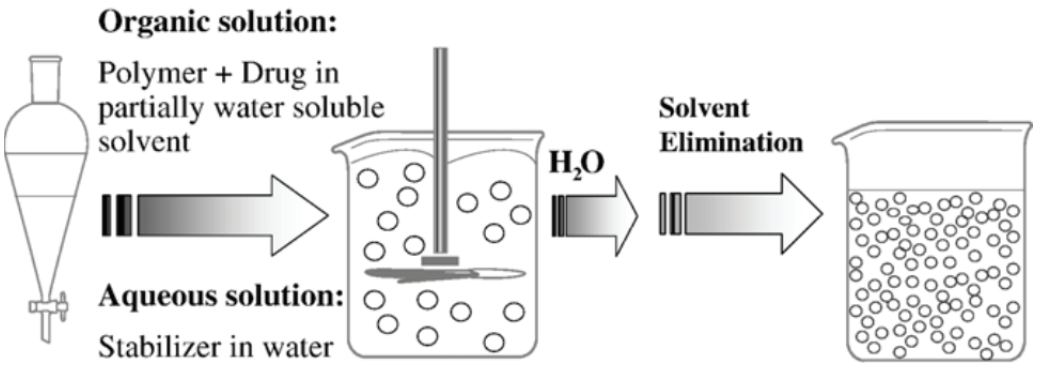

Figure 2: Emulsion diffusion technique (Pinto Reis et al., 2006)

Table 3: Applications of the emulsion diffusion method

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Articaine	PCL	-	-	Campos et al., 2013
Omeprazole	Eudragit L 100-55	0.256.3- 0.337	8.92 - 16.53	Hao et al., 2013
Curcumin	Polyurethane and polyurea	0.216- 4.901	-	Souguir et al., 2013
Matricaria recutita L. extract	PEG-PBA-PEG	0.186- 0.446	-	Esmaeili et al., 2011
Bovine serum albumin	Chitosan	81-98	-	Karnchanajindanun et al., 2011
Alendronate	PLGA	0.145	-4.7	Cohen-Sela et al., 2009
An oligonucleo-tide	PLA	0.390	-	Delie et al., 2001

3.3 Simple Emulsion evaporation (SEE)

The SEE technique is widely used in the field of particulate carriers' development. This method was first developed by (Vanderhoff et al., 1979). It consists on the formation of a simple emulsion followed by the evaporation of the organic solvent. Subsequent precipitation of the polymer allows the obtaining of the particles (Figure 3). Practically, for oil in water emulsion method, the polymer is dissolved in a volatile and non miscible organic solvent such as chloroform, ethylacetate or dichloromethane. This organic phase, in which the drug and the polymer are dissolved, is then dispersed by high speed homogenization or by sonication in an aqueous phase containing a surfactant. Once an oil-in-water (o/w) emulsion is obtained, the evaporation of the organic solvent permits the precipitation of the polymer and thus, the formation of the particles. As it can be seen in Table 4, SEE is generally used for the encapsulation of hydrophobic drugs (O'Donnell and McGinity, 1997). The evaporation of the organic solvent is obtained by moderately stirring the emulsion at room temperature or under high temperature and low pressure conditions. The obtained particles can be then harvested by ultracentrifugation or filtration, then washed and lyophilized. Membrane technology was also used to prepare particles by the simple emulsion technique (Doan et al., 2011). Another alternative of the technique is the use of water in oil emulsion method that is suitable for the encapsulation of hydrophilic active molecules. Particulate carriers are obtained after evap-

oration of the water phase which causes the precipitation of the hydrophilic polymer (Banerjee et al., 2012). Parameters that have to be managed include organic phase to water phase ratio, nature of the surfactant and its concentration, stirring rate, polymer amount and evaporation rate. Decreasing the organic solvent volume resulted generally in a decrease of particle size (Budhian et al., 2007). Particle size could also be decreased by increasing surfactant amount (Valot et al., 2009; Manchanda et al., 2010; Khaled et al., 2010; Su et al., 2009), increasing stirring rate (Su et al., 2009; Lee et al., 2012; Avachat et al., 2011; Yadav and Sawant, 2010) or increasing aqueous phase volume (Adibkia et al., 2011). However, an increase of polymer amount generally increases particles' size (Doan et al., 2011; D'Aurizio et al., 2011; Adibkia et al., 2011; Agnihotri and Vavia, 2009). Table 4 shows the applications of the SEE technique in drug delivery. Polyesters were widely used for the encapsulation of hydrophobic drugs.

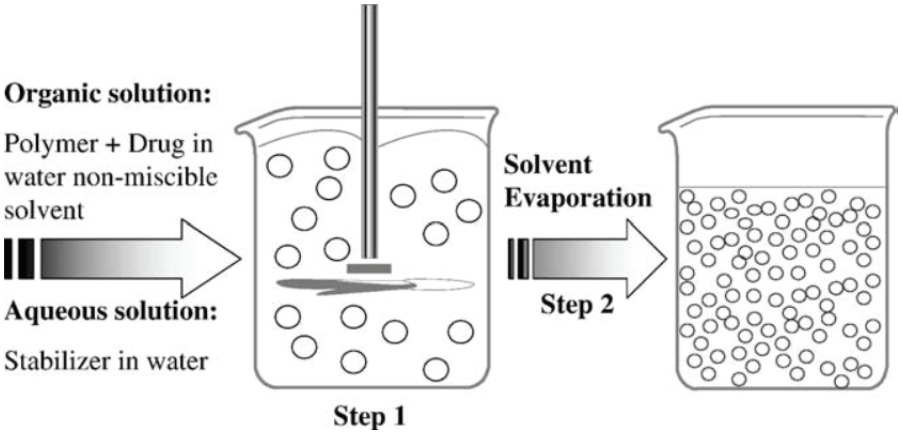

Figure 3: Simple emulsion solvent evaporation (Pinto Reis et al., 2006)

Table 4: Applications of simple emulsion solvent evaporation technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Curcumin	PLGA and PLGA-PEG	0.161-0.152	-	Khalil et al., 2013
Efavirenz	PCL and Eudragit® RS 100	0.083-0.219	53	Seremeta et al., 2013
Human amylin	PCL	0.202	-	Guerreiro et al., 2012
Azithromycin	PLGA	14.11-15.29	-	Li et al., 2012
Teniposide	PLGA	0.113-0.135	-36.6-(-23.1)	Mo et al., 2012
Camptothecin	PCL-PEG-PCL	4.2-5.4	-	Dai et al., 2011
Naproxen	PLGA	352-824	-	Javadzadeh et al., 2010
Doxorubicin	PLGA	0.137-0.164	-12.3-(-9.9)	Manchanda et al., 2010
Dexamethasone	PLGA	5.18-7	-	Rawat and Burgess, 2010
Ibuprofen	Eudragit RSPO	14-51.1	-	Valot et al., 2009

3.4 Double emulsion evaporation (DEE)

Double emulsion technique is suitable for the encapsulation of hydrophilic molecules (see Table 5 and Figure 4). Generally, the method consists on the dispersion of an aqueous phase in a non miscible organic solvent to form the first emulsion (W1/O). This dispersion is performed under high shear homogenization or low power sonication for a short time. This step is followed by the dispersion of the obtained emulsion in a second aqueous phase containing a hydrophilic emulsifier. Again, homogenization could be carried under high shear homogenization or with a sonication probe. When sonication is used, it must be performed at low power and within a short period of time to not break the first emulsion (Giri et al., 2013). After the formation of the multiple emulsion, evaporation of the volatile organic solvent under low pressure (by a rotary evaporator) or at ambient temperature allows the obtaining of the particulate carriers (Figure 4). There are other types of multiple emulsions like w/o/o or o/w/o (Giri et al., 2013). A lot of parameters may influence the properties of the obtained particles such as, relative phases' ratio (Khoee et al., 2012), amount of the polymer, its nature and molecular weight (Zambaux et al., 1998; Péan et al., 1998; Van de Ven et al., 2011), nature of the surfactants and their amounts (Zhao et al., 2007; Khoee and Yaghoobian, 2009; Dhanaraju et al., 2004), homogenization speed (Eley and Mathew, 2007; Basarkar et al., 2007), the composition of the external phase (Péan et al., 1998; Tse et al., 2009) and evaporation speed (Khoee et al., 2012). Operating conditions may also influence strongly encapsulation efficiency (Tse et al., 2009; Billon et al., 2005; Silva et al., 2013; Zhou et al., 2013; Karataş et al., 2009; Hachicha et al., 2006; Al-Kassas, 2004; Cun et al., 2011; Gaignaux et al., 2012; Cun et al., 2010). Membrane technique and microfluidic devices were also used to prepare particulate carriers by the DES method (Vladisavljević and Williams, 2008; van der Graaf et al., 2005).

3.5 Spray drying

Spray drying is a simple process which gained too much interest due to its cost-effectiveness and scalability (Sou et al., 2011). Practically, a polymer containing drug solution is atomized and sprayed into a drying chamber where droplets are dried by heated air (See Figure 5). Reduction of droplets' size that follows atomization allows the obtaining of an enormous surface area between droplets and the drying gas. The subsequent precipitation of the polymer permits the encapsulation of the drug within the obtained particulate carriers. The evaporation of the solvent occurs within a very short period of time. Consequently, the materials never reach the inlet temperature of drying gas. This is very attractive for encapsulating heat-sensitive drug molecules like proteins (Cal and Sollohub, 2010; Sollohub and Cal, 2010; Prata et al., 2013). Many operating conditions could influence the properties of the obtained particles. Parameters to be controlled include the drying air temperature and humidity (Bruschi et al., 2003), the rate and fluid dynamics of the air flow, the atomization process (Droplet size, spray rate, spray mechanism) and the composition of ingredients and excipients in the feeding solution (Rattes and Oliveira, 2007). PLA (Baras et al., 2000; Gander et al., 1996; Sastre et al., 2007; Muttil et al., 2007), PLGA (Wang and Wang, 2002; Mu and Feng, 2001; Castelli et al., 1998; Bittner et al., 1999; Prior et al., 2000; Conti et al., 1997), PCL, methacrylate polymers (Esposito et al., 2002; Año et al., 2011; Cruz et al., 2010; Hegazy et al., 2002; Raffin et al., 2008) and chitosan (He et al., 1999; Giunchedi et al., 2002; Cevher et al., 2006) are among the most used polymers in spray-drying method. As Table 6 shows, the technique allowed the obtaining of mainly microparticles bearing better drug solubility and sustained release.

Table 5: Applications of the double emulsion technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Vancomycin	PLGA	0.450-0.466	-7.2-(-3.5)	Zakeri-Milani et al., 2013
Prostaglandin E1	PLGA	7-22.5	-	Gupta and Ahsan, 2011
Deoxyribonuclease I	PLGA	0.190-0.349	-	Osman et al., 2011
S. equi antigens	PCL	0.242-0.450	-53.1-38.7	Florindo et al., 2009
Hepatitis B surface antigen	PLGA	1-5	0.51-14	Thomas et al., 2009
Plasmid DNA	PLGA	1.9-4.6	-24.6-(-22.9)	Tse et al., 2009

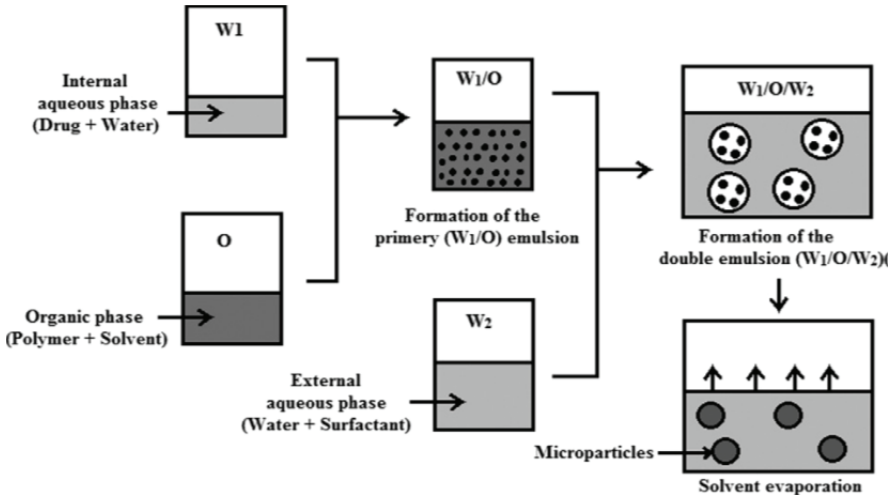

Figure 4: Double emulsion solvent evaporation technique (Giri et al., 2013)

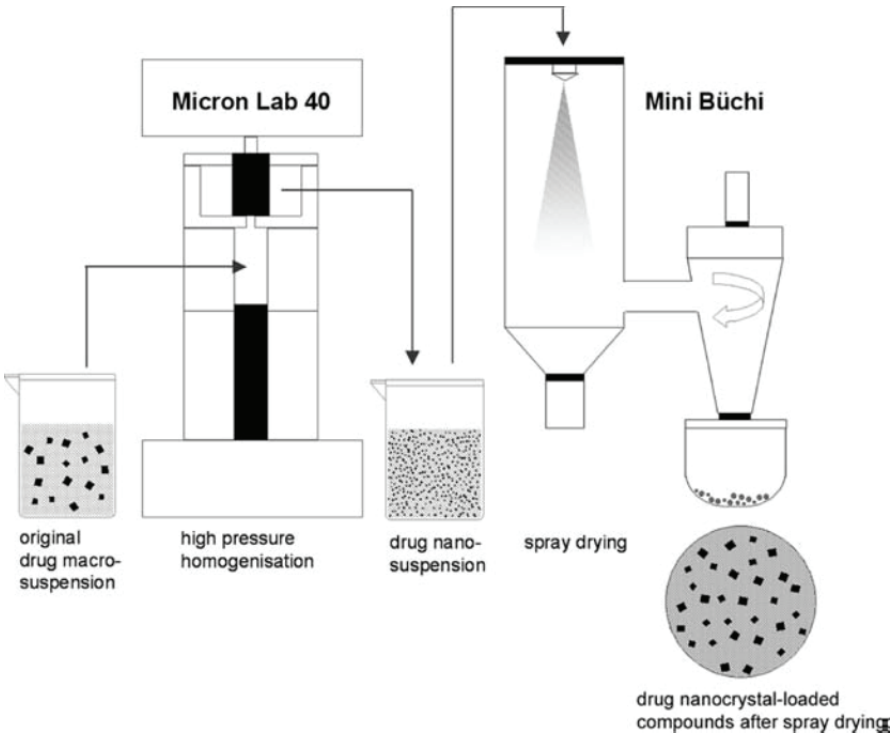

Figure 5: The spray drying method (Pinto Reis et al., 2006)

Table 6: Applications of the spray drying technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Nimodipine	PLGA	1.9-2.37	-	Bege et al., 2013
Theophylline	Eudragit RS30D	< 60	-	Garekani et al., 2013
Ofloxacin	PLA	2.6-4.9	-	Palazzo et al., 2013
Sodium diclofenac	PGA-co-PDL PEG-PGA-co-PDL and mPEG-co-(PGA-co-PDL)	2.3 3.9 2.5	-32.2 -29.9 -31.2	Tawfeek, 2013
Sodium fluoride	Chitosan	3.4-5.3	-	Keegan et al., 2012
Plasmid	Chitosan	2.5-11.7	-	Mohajel et al., 2012
Heparin	PLGA	2.5-3.8	-63.5 - (-28.2)	Yildiz et al., 2012
Alendronate	Eudragit® S100	13.8	-	Cruz et al., 2010
Zolmitriptan	Chitosan glutamate and Chitosan base	2.6-9.4	-	Alhalaweh et al., 2009
Triamcinolone	PLGA	0.5-1.5	-	da Silva et al., 2009
Acyclovir	Chitosan	18.7-34.9	-	Stulzer et al., 2009

3.6 Supercritical fluid technology (SFT)

In the recent years, novel particle formation techniques using supercritical fluids (SCF) have been developed in order to overcome some of the disadvantages of conventional techniques that are: (1) poor control of particle size and morphology; (2) degradation and lost of biological activity of thermo sensitive compounds; (3) low encapsulation efficiency and (4) low precipitation yield (Santos et al., 2013). Moreover, SFT presents the main advantage of not requiring the use of toxic solvents. In fact, SCF based technologies have attracted enormous interest for the production of microparticles and nanoparticles (Table 7), since their emergence in the early 1990s (Sanli et al., 2012).

The supercritical state is achieved when a substance is exposed to conditions above its critical pressure and temperature. In such conditions, the fluid will have liquid-like density and, thus, solvating properties that are similar to those of liquids and, at the same time, gas-like mass transfer properties. Carbon dioxide (CO_2) is the most commonly used critical fluid. In fact, CO_2 is nontoxic, nonflammable and easy recyclable. Moreover, CO_2 has moderate critical parameters of CO_2 (a critical pressure of 7.4 MPa and a critical temperature of 304.1 K) and low price and is highly available which makes it very attractive from an economical point of view and also for the processing of labile compounds (Elizondo et al., 2012). Supercritical fluid technology methods can be divided in four methods which are rapid expansion of supercritical solution (RESS), Particles from gas saturated solutions (PGSS), gas antisolvent (GAS) and supercritical antisolvent process (SAS). These methods depend on whether CO_2 was used as a solvent, a solute or an antisolvent. Figure 6 shows the experimental set up of the RESS technique. In the RESS technique, the drug and the polymer are first dissolved in supercritical CO_2 in high pressure chamber. The subsequent passing of the solution through a nozzle results in a rapid decrease of the pressure and thus, a precipitation of the drug particles embedded in the polymer matrix and their recovery in the extraction unit (Byrappa et al., 2008). Many parameters such as the density of the SCF (Pressure and temperature of supercritical fluid) (Kalani and Yunus, 2012), flow rate of drug-polymer solution and/or CO_2 and formulation variables (Martin et al., 2002) could influence the size of the obtained particles. Table 7 shows that SFT was used for

the processing of nanoparticles and microparticles mainly based on polyesters.

3.7 Ionic gelation (IG)

IG method is used mainly with natural hydrophilic polymers to prepare particulate carriers. These polymers include gelatin, alginate, chitosan and agarose. IG has the advantage of not using organic solvents. The technique is based on the transition of the polymer from liquid state to a gel (Figure 7). For instance, gelatin based particles are obtained after the hardening of the droplets of emulsified gelatin solution. The particles are obtained after cooling gelatin emulsion droplets below the gelation point in an ice bath. For alginate, however, particles are produced by drop-by-drop extrusion of the sodium alginate solution into the calcium chloride solution. Sodium alginate is, in fact, a water-soluble polymer that gels in the presence of multivalent cations such as calcium. Chitosan particles are prepared by spontaneous formation of complexes between the positively charged chitosan and polyanions (tripolyphosphate or gelatin) or by the gelation of a chitosan solution dispersed in an oil emulsion (Mahapatro and Singh, 2011). Figure 7 illustrates the gelation mechanism of polysaccharides. At high temperatures, a random coil conformation is assumed. With decreasing temperature, the aggregation of double helices structure forms the physical junctions of gels (Rees and Welsh, 1977). Table 8 displays some recent applications of IG. This technique has been mainly used to prepare chitosan nanoparticles.

Table 7: Applications of the SCF technology

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Hydrocortisone acetate	PLGA	1-5	-	Falco et al., 2013
17α-methyltestosterone	PLA	5.4-20.5	13.9 - 67.7	Sacchetin et al., 2013
Paracetamol	PLA	0.301-1.461	-	Kalani and Yunus, 2012
5-fluorouracil	PLLA-PEG/PEG	0.175	-	Zhang et al., 2012
Human growth hormone	PLGA	93	-	Jordan et al., 2010
Azacytidine	PLA	2	-	Argemí et al., 2009
Bovine serum albumin	PLA	2.5	-	Kang et al., 2009
Retinyl palmitate	PLA	0.040-0.110	-	Sane and Limtrakul, 2009
Indomethacin	PLA	2.35	-	Kang et al., 2008

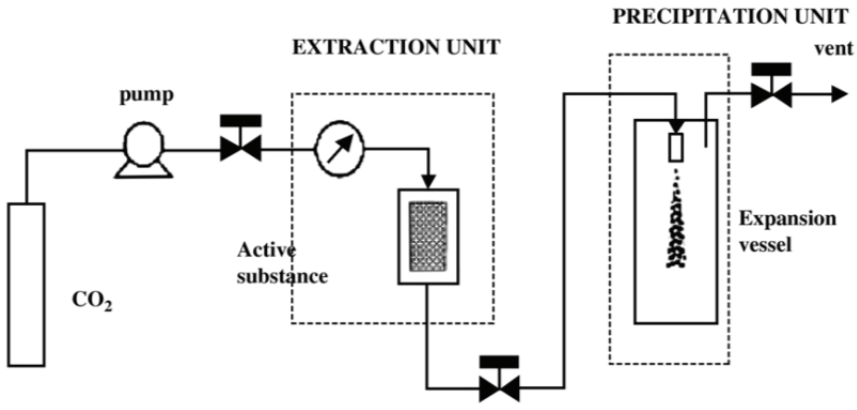

Figure 6: Schematic presentation of the experimental set up for the RESS process (Byrappa et al., 2008)

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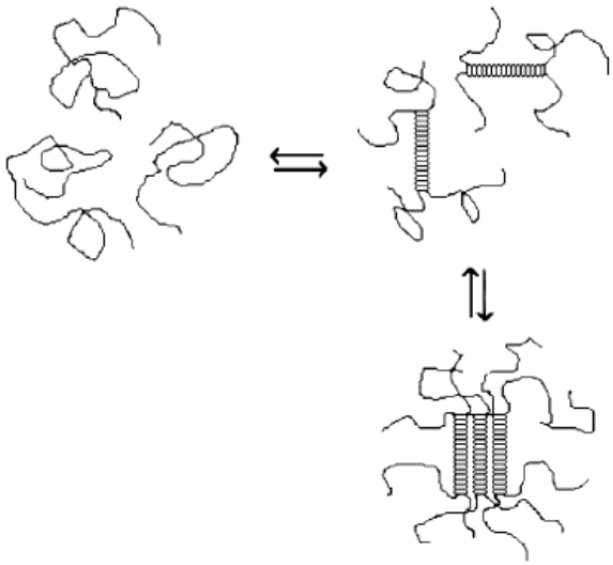

Figure 7: Gelation mechanism of polysaccharides in water (Guenet, 1992)

Table 8: Some applications of the ionic gelation technique

Encapsulated molecule	Polymer	Size (µm)	Zeta potential (mV)	Reference
Articaine hydrochloride	Alginate/chitosan	0.340-0.550	-22 - (-19)	de Melo et al., 2013
TNF-α siRNA	Trimethyl chitosan-cysteine	0.146	25.9	He et al., 2013
Paclitaxel	O-carboxymethyl chitosan	0.130-0.180	-30 - (-12)	Maya et al., 2013
pDNA	Chitosan	0.153-0.403	46.2-56.9	Cadete et al., 2012
Gemcitabine	Chitosan	0.095	-	Derakhshandeh and Fathi, 2012
Dexamthasone sodium phosphate	Chitosan	0.256-0.350	-	Doustgani et al., 2012
Itraconazole	Chitosan	0.190-0.240	11.5-18.9	Jafarinejad et al., 2012
5-fluorouracil and leucovorin	Chitosan	0.040-0.097	25.6-28.9	Li et al., 2011
Insulin	Chitosan and arabic gum	0.172-0.245	35.7-43.4	Avadi et al., 2010
CKS9 peptide sequence	Chitosan	0.226	-	Yoo et al., 2010

3.8 Layer by layer

Polyelectrolyte self assembly is also called layer-by-layer (LbL) assembly process. The earliest technology was based on the assembly of colloidal particles on a solid core (Iler, 1966). From the 1990s, applications were expanded. LbL allowed, in fact, the assembly of polyelectrolyte films using biopolymers, proteins, peptides, polysaccharides and DNA (Powell et al., 2011). This approach was first developed by Sukhorukov et al. (Sukhorukov et al., 1998). Polyelectrolytes are classified according to their origin. Standard synthetic polyelectrolytes include poly(styrene sulfonate) (PSS), poly (dimethyldiallylammonium chloride) (PDDA), poly(ethylenimine) (PEI), poly(N-isopropyl acrylamide (PNIPAM), poly-

(acrylic acid) (PAA), poly (methacrylic acid) (PMA), poly(vinyl sulfate) (PVS) and poly(allylamine) (PAH). Natural polyelectrolytes include nucleic acids, proteins and polysaccharides such as, alginic acid, chondroitin sulfate, DNA, heparin, chitosan, cellulose sulfate, dextran sulfate and carboxymethylcellulose (de Villiers et al., 2011). The obtained particles are vesicular and are called polyelectrolyte capsules. Assembly process is based on irreversible electrostatic attraction that leads to polyelectrolyte adsorption at supersaturating polyelectrolyte concentrations. Other interactions such as, hydrogen bonds, hydrophobic interactions and Van der Waals forces were also described (de Villiers et al., 2011). A colloidal template that serves to the adsorption of the polyelectrolyte is also needed. The commonly used cores for the formulated particles are derived from stabilized colloidal dispersions of charged silica, charged poly(styrene) spheres, metal oxides, polyoxometalates and conducting liquid crystalline polymers. Carrier systems can be functionalized with stimuli-responsive components that respond to temperature, pH and ionic strength. The polymers/colloids used in LbL technique can also be functionalized to alter their properties preceding layer by layer assembly. Experimental parameters that have to be managed include coating material concentration, ion concentration and the pH of the medium (Vergaro et al., 2011). Polymer assembly occurs after incubation of the template in the polymer solution or by decreasing polymer solubility by drop-wise addition of a miscible solvent (Radtchenko et al., 2002). This procedure could be repeated with a second polymer to allow sequential deposition of multiple polymer layers (Figure 8). LbL presents advantages over several conventional coating methods: (1) simplicity of the process and equipment; (2) its suitability for coating most surfaces; (3) availability and abundance of natural and synthetic colloids; (4) flexible application to objects with irregular shapes and sizes; (5) formation of stabilizing coats and (6) control over the required multilayer thickness (de Villiers et al., 2011). Table 9 contains some recent applications of LbL technique.

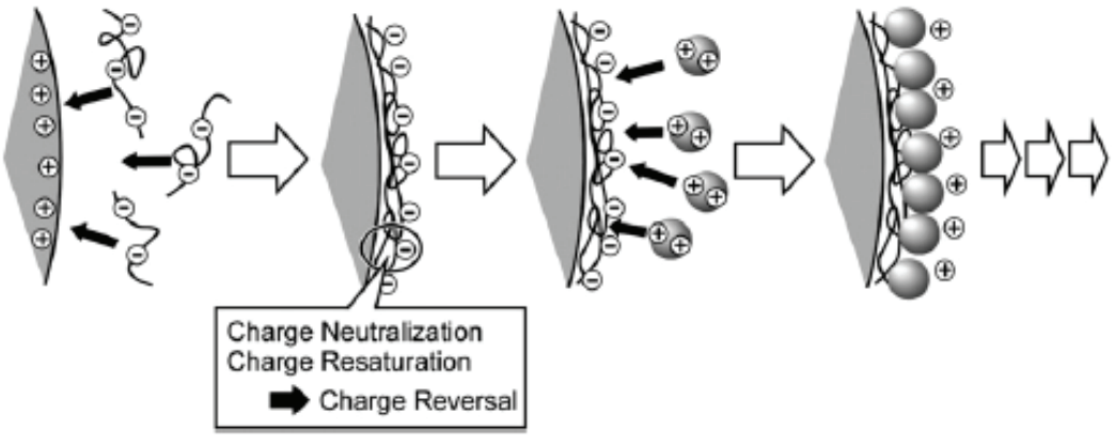

Figure 8: The layer by layer technique based on electrostatic interaction (Ariga et al., 2011)

Table 9: Applications of the layer-by-layer technique

Active	Polyelectrolytes	Size (µm)	Zeta potential (mV)	References
Kaempferol	Sodium Alginate and protamine sulfate	0.161	- 8.9	Kumar et al., 2012
Designed peptide DP-2015	Poly-l-glutamic acid and poly-l-lysine	-	-	Powell et al., 2011
5-fluorouracil	Poly(L-glutamic acid) and chitosan	1	25-40	Yan et al., 2011
Plasmid DNA	Plasmid DNA and reducible hyperbranched poly(amidoamine) or polyethylenimine	-	-	Blacklock et al., 2009
Artemisinin	Alginate, gelatin and chitosan	0.806	-33	Chen et al., 2009
Insulin	Glucose oxidase and catalase	6	-	Qi et al., 2009
Heparin	Poly(styrene sulfonate) and chitosan	1	-10.4	Shao et al., 2009
Acyclovir	Poly(vinyl galactose ester-co-methacryloxyethyl trimethylammonium chloride) and poly(sodium 4-styrenesulfonate)	-	-	Zhang et al., 2008a
Propranolol hydrochloride	Poly(vinyl galactose ester-*co*-methacryloxyethyl trimethylammonium chloride) and Poly(sodium 4-styrenesulfonate)	5-15.6	-	Zhang et al., 2008b

CONCLUSION

Encapsulation of active molecules is a crucial approach that has been widely used for many biomedical applications. It permits enhancement of bioavailability of molecules, sustained delivery, passive or active targeting and decrease of toxicity and side effects. These formulations can render some active molecules more suitable for a specific route such as the delivery of proteins by the oral route or the delivery of some drugs via the blood brain barrier. Thus, they enhance efficiency, patient compliance and allow successful management of diseases. Many biodegradable and biocompatible polymers were investigated. The choice of the technique and the suitable polymer is a crucial step. It depends on the physicochemical properties of the drug to be encapsulated. The management of operating conditions is also a hard task to monitor particles' properties and to enhance drug loading. Recent research works are focusing on active targeting by the coating the carriers by biomolecules that specifically recognize a well-defined cell receptor. One can also notice a shift for more 'intelligent' drug delivery systems. Responsive materials, for example, react to a specific physiological stimulus such as a variation of pH to release the encapsulated drug. Other thermo-sensitive materials deliver drugs at a specific temperature. It can be noted also that more attention is paid to safer methods that avoid the use of organic solvents (RESS) or to techniques that provide better reproducibility and easy scalability (microfluidics and membrane emulsification technology), which could be attractive for industrial processing.

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II.2. Encapsulation via double emulsification process

Summary

Double emulsions also termed as multiple emulsion or "emulsions of emulsions" are systems in which the droplets of dispersed themselves comprises of one or more smaller dispersed droplets. In this system, two liquid phases are separated by a third non miscible liquid phase. They can be composed of aqueous droplets encapsulating oil drops, or vice versa.

The entrapment of water-soluble drug in polymeric micro- and nanoparticles with high loading remains a challenge because of rapid diffusion of the compound into the external aqueous continuous phase. The double emulsion solvent evaporation technique can be used, in order to overcome the problem of inefficient encapsulation of hydrophilic drugs. The most commonly used type is $W_1/O/W_2$ double emulsion solvent evaporation method, in which emulsification is performed in two step. First, the primary emulsion (W_1/O) is prepared by homogenization of inner aqueous phase (W_1) with organic phase (polymer solution). Then, the primary emulsion is dispersed in a second aqueous phase containing suitable stabilizer (s) and homogenizes to form double emulsion. The formation of emulsion is followed by evaporation of volatile organic solvent from the dispersed phase leading to a point of insolubility and precipitation of the polymer encapsulating the active material. The outer aqueous phase acts as dispersion medium. The volatile solvent may be eliminated by stirring at ambient temperature or under reduced pressure by rotary evaporator depending upon the nature of organic solvent.

The organic solvent used for the encapsulation of drugs via double emulsion method should be of low boiling point in order to facilitate the removal of residual solvent from final particulate dispersion. Commonly used solvents are: dichloromethane, chloroform, methylene chloride, propylene carbonate and ethyl acetate. Among them, methylene chloride and ethyl acetate are very frequently used. Compared to the more hydrophobic methylene chloride, ethyl acetate usually exerts a less deteriorative effect on bioactivity of the entrapped peptides and proteins. Though, most of the researchers still prefer methylene chloride as the organic solvent due to its ability to dissolve huge amounts of biodegradable polymers, low solubility in water (2.0%, w/v) and low boiling point (39.8 °C), thus, facilitating its removal by evaporation. Conversely, the relatively high solubility in water (8.7%, w/v) and the high boiling point (76.7 °C) of ethyl acetate limited its application in $W_1/O/W_2$ double emulsion technique. The comparatively high solubility of ethyl acetate in water contributes to a fast diffusion of ethyl acetate from oil droplets into the external aqueous phase during the second step of emulsification

process, which leads to polymer precipitation rather than the formation of micro- and nanoparticles.

The performance of the polymeric encapsulation via double emulsion method depends on several factors such as biodegradable polymer nature (polymer composition and molecular weight), physicochemical properties organic solvents, type and concentration of surfactant, etc. Biodegradable polymers have the ability to degrade into nontoxic components; therefore, they have been used vastly in drug delivery system for encapsulation of pharmaceuticals and pharmaceuticals. Commonly used biodegrade polymers in double emulsion process include, poly (lactic acid) (PLA), poly lactic-co-glycolic acid (PLGA) and polycaprolactone (PCL), which are being widely researched for encapsulation various active drugs, genes and macromolecules. PCL is a nontoxic, biodegradable and biocompatible polymer, and possess low glass transition temperature and melting point (60 °C) and its metabolites are eliminated from the body by innate metabolic process. It has been widely studied for encapsulation of drugs for control drug delivery system due to its compatibility with vast range of drugs and its slow degradation to release drug for long time. PLGA is another biocompatible and biodegradable polymers commonly used in encapsulation via double emulsion method. After its approval by FDA for use in humans, it has been a popular choice for drug delivery. It degrades slowly into the biocompatible products of lactic and glycolic acid via hydrolysis and thus releases the encapsulated agents slowly over a long period of time. This polymer is available in different PLGA/PLA ratio. As lactic acid is more hydrophobic than glycolic acid and, thus, lactic acid-rich PLGA copolymers are less hydrophilic, and degrade very slowly. PLA polymers are also approved by FDA in humans and have been frequently used for encapsulation in drug delivery system. It belongs to the most promising category of biodegradable polymers having excellent mechanical properties, good biocompatibility, and low toxicity. It is semi-crystalline polymer with a melting point of about 180 °C, glass transition temperature of about 55 °C and tensile strength of 50–70 MPa and commonly prepared by ring-opening polymerization (ROP). Beside many advantages of biodegradable PLA and PLGA polymers in controlled drug delivery systems, they have certain drawbacks such as a risk of toxicity and immunogenicity because of their acidic by-products when used for long duration.

This method has been used widely for encapsulation of different pharmaceuticals and biopharmaceuticals. Various types of biomolecules such as nucleic acids proteins and peptides

have the challenge of their effective and efficient delivery to the target site without its degradation. Due to their water solubility, they tend to diffuse into continuous aqueous phase during emulsification process, which leads to low entrapment efficiency of bioactive molecule. Moreover, the negative charged biomolecules are unable to pass through the cell membrane properly and can be denatured by different enzymes such as proteases and nucleases. Therefore, different approaches have been used for encapsulation of nucleic acid in order to protect it against degradation, facilitating its intracellular penetration enhancing its delivery to intended site and controlling the rate of release. These apaches include, emulsion solvent evaporation, coacervation, spray drying and double emulsion techniques. However double emulsion technique is considered one of the most appropriate method, especially for encapsulation of hydrophilic nucleic acid. This technique is reproducible and can be scale up for large batches. Beside several advantages, double emulsion process has a shortcoming of shear force used for homogenization of nucleic acid's solution in the organic phase. Which can damage the integrity of biomolecules, thus leads to loss of its bioactivity. Moreover, the organic solvents can damage the structure of nucleic acid during emulsification process. This damage can be minimized by condensation of nucleic acid with cationic polymers, in order to reduce its size and maintain its supercoiling structure, and thus preserve its biological activity.

Several categories of pharmaceuticals have been encapsulated by double emulsion method such as anticancer drugs, antibiotics and anti-inflammatory drugs. The anticancer are encapsulated into biodegradable polymer in order to provide prolonged release of drug in controlled manner and to target the drug to specific cancerous tissues. Moreover co-delivery of both hydrophilic and hydrophobic drug and diagnostic agent simultaneously (theranostic) can be possible by this technique. These processes are also useful for effective encapsulation of highly water soluble antibiotics and for sustained release of antibiotics. Similarly the anti-inflammatory medicaments can be encapsulated in order to diminish their systemic side effects and to reduce the chances of possible drug interaction with concurrent medications. During encapsulation of various drugs via double emulsion method, the ratio of aqueous phase to oil phase has a significant effect on double emulsion stability. It has been established that, an emulsion with ratio of 1:10 (water: oil) is more stable than ratio of 1:5 (water: oil). Similarly, the emulsion of smaller particle size can be prepared with water: oil ratio of 1:30 as compared to ratio of 1:10

(water: oil), however beyond water: oil ratio of 1:20 a decrease in encapsulation efficiency has also been observed, thus water: oil ratio in between 1:10 and 1: 20 are widely used.

Beside many applications in controlled drug delivery and encapsulation of hydrophilic drugs, double emulsion has also several applications in theranostics. Theranostic is an approach in which agents are developed for simultaneous diagnosis and treatment of various diseases. It has been the field of extensive investigations in recent years for biomedical applications. These multifunctional theranostic agents allow for feedback mechanism to establish the localization of drug, release of drug, disease phase and efficacy of the treatment. For example, Yang et al fabricated polymer wormlike vesicles loaded with loaded with superparamagnetic iron oxide (SPIO) nanoparticles and anticancer drug doxorubicin (DOX) for targeted cancer therapy and MR imaging. The calculated SPIO nanoparticles (NPs) loading content in the vesicles was about 48.0 wt. %, while The DOX loading level for these vesicles was about 9.0 wt. %. This theranostic vesicle nanocarrier system was established to be very efficient, which can provide controlled and targeted drug delivery to the tumor as well as it can be used as an efficient MRI contrast agent, thus providing targeted cancer therapy and diagnosis simultaneously.

Double emulsion solvent evaporation techniques used for drug encapsulation

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Abstract

Double emulsions are complex systems, also called "emulsions of emulsions", in which the droplets of the dispersed phase contain one or more types of smaller dispersed droplets themselves. Double emulsions have the potential for encapsulation of both hydrophobic as well as hydrophilic drugs, cosmetics, foods and other high value products. Techniques based on double emulsions are commonly used for the encapsulation of hydrophilic molecules, which suffer from low encapsulation efficiency because of rapid drug partitioning into the external aqueous phase when using single emulsions. The main issue when using double emulsions is their production in a well-controlled manner, with homogeneous droplet size by optimizing different process variables. In this review special attention has been paid to the application of double emulsion techniques for the encapsulation of various hydrophilic and hydrophobic anticancer drugs, anti-inflammatory drugs, antibiotic drugs, proteins and amino acids and their applications in theranostics. Moreover, the optimized ratio of the different phases and other process parameters of double emulsions are discussed. Finally, the results published regarding various types of solvents, stabilizers and polymers used for the encapsulation of several active substances via double emulsion processes are reported.

Keywords: Double emulsion, Solvent evaporation, Encapsulation, Theranostics, Control release, Drug delivery.

1. Introduction

Nowadays, scientific advanced technologies are being directed towards evolution of innovative pharmaceutical products. The conventional therapies are progressively supplemented by more adaptable and well refined drug deliveries by the exploration of these advanced diversified technologies. Special focus is paid on tackling the restrictions associated with traditional drug delivery. Some of the most common obstacles encountered are the low bioavailability, poor stability, bitter taste and unpleasant odor of certain active agents. Here, encapsulation plays a vital role in overcoming the aforementioned obstacles. Premature degradation of active agents especially of proteins and peptides can be prevented by their encapsulation. Furthermore, encapsulation technologies also aid in achieving controlled and targeted release formulations. Colloidal carriers find immense applications in biomedical and biotechnology field. Various kinds of colloids employed in medicine are dendrimers, block ionomer complexes, polymeric biodegradable nanoparticles (NPs), polymeric micelles, liposomes (Laouini et al., 2012; Naseer et al., 2014), nanotubes, nanorods (Wang et al., 2012) and quantum rods.

Recently, polymers are increasingly used to constitute biodegradable particles. Such particles serve as drug reservoirs which bring not only local therapeutic effect but also deliver drugs, genes and vaccines to target organs for site specific delivery (Mohsen Jahanshahi, 2008; Zafar et al., 2014). Polymers are also employed in regenerative medicines and tissue engineering. Polymer selection is primarily based on toxicity and final application of the polymer. Biodegradable polymer encapsulated drugs show superb attributes e.g. non-toxicity and stability in blood. Moreover, polymeric materials permit modification in a) physicochemical characteristics (e.g. hydrophobicity, zeta potential), b) drug release properties (e.g. delayed, prolonged, triggered) and c) biological behaviour (e.g. bioadhesion, improved cellular uptake) of the NPs (Galindo-Rodriguez et al., 2005; Kumari et al., 2010; des Rieux et al., 2006). Examples of commonly used biodegradable polymers for encapsulation include poly (lactic acid), poly(glycolic acid), and poly (lactic-co-glycolic acid) (PLGA). Biodegradable polymers have the advantage that the release of loaded drug depends mainly on the degradation kinetics of the polymers used e.g. the release rate from PLGA carriers can be controlled by altering the lactic acid and glycolic acid ratio and molecular weight (Ye et al., 2010). Successful encapsulation of drugs like paclitaxel, 9-nitrocamptothecin, cisplatin, insulin, dexamethasone, estradiol,

progesterone, tamoxifen, tyrphostins and haloperidol (Kumari et al., 2010) has been done by using various natural and synthetic polymers.

Several encapsulation processes have been used in order to protect and to transport active molecules. These techniques can be divided in two major classes: (i) chemistry based processes e.g. emulsion polymerization, miniemulsion polymerization and interfacial polymerization, (ii) physicochemical processes e.g. multiple emulsion techniques, spray drying, emulsion solvent diffusion and layer by layer process. Different pharmaceutical carriers such as microparticles, nanoparticles and liposomes can be obtained by these techniques (Miladi et al., 2013). Microencapsulation by solvent evaporation is mostly used in pharmaceutical industries to get controlled release formulations. Different methods are available to use microencapsulation by solvent evaporation technique. The selection of a method that will give adequate drug encapsulation usually depends on the hydrophilicity or hydrophobicity of the active molecules (Li et al., 2008). Oil-in-water (o/w) method is generally adopted for the encapsulation of insoluble or poorly water soluble active agents. Few examples of hydrophobic active agents that have been encapsulated via this technique include Cisplatin (Verrijk et al., 1992), 5-Fluorouracil (Boisdron-Celle et al., 1995), Lidocaine (Chung et al., 2001) and Progesterone (Aso et al., 1994; Bums et al., 1993). However, this technique fails when it comes to encapsulation of highly hydrophilic agents. This is because the active agent may diffuse into the continuous phase during the formulation or it may not get dissolved in the organic solvent. Multiple emulsions play a pivotal role in such cases. The most common type of multiple emulsions is water-in-oil (w/o/w) emulsion. Most hydrophilic drugs have been encapsulated via (w/o/w) method (Crotts and Park, 1998; Okochi and Nakano, 2000; Sinha and Trehan, 2003). The water soluble agent is solubilized within the inner W_1 phase of the emulsion which then shows prolonged drug release, lesser toxic effects (Nakhare and Vyas, 1996) and high encapsulation efficiency of the active agent. For this reason, proteins have been widely encapsulated by w/o/w emulsion system. The stability and release properties of double emulsions can be greatly improved by a change in the type and concentration of stabilizer employed in the system. Combining targeted delivery and prolonged drug release by using double emulsion system presents tremendous benefits in cancer therapy. Many advantages are associated with the use of double emulsion systems. Such systems are biocompatible, biodegradable and versatile with respect to different oils and emulsifiers being employed. Moreover, both hydrophobic and hydrophilic kinds of drugs can be

encapsulated (separately and simultaneously) and protected. However, some difficulties are also linked with multiple emulsions such as difficulty in formulation, bulky and susceptibility to different routes of physical and chemical degradation. Various attempts have been made by researchers to improve the stability of multiple emulsions. Some of these efforts include surfactant concentration modulation, interfacial complexation, polymerization gelling, additives in different phases, steric stabilization and pro-emulsion approach (Garti, 1997; Garti and Aserin, 1996; Hino et al., 2000). The objective of this work is to present a comprehensive literature review on double emulsion technique. The review begins with a brief description of double emulsion and a comprehensive history of this technique. It progresses on to discuss the mechanism involved in formulation of a double emulsion. This review also reports the different polymers, stabilizers, surfactants, drugs encapsulated and average particle size obtained in different studies via this technique.

2. What is a double emulsion?

The first paper on double emulsion dates back 89 years (Seifriz, 1924), but detailed investigation on double emulsions was started at the end of 1970s. We can find several reviews on multiple emulsions, which come primarily from three research groups namely: Florence and Whitehill, (Florence and Whitehill, 1981a, 1982), Matsumoto et al. (Matsumoto et al., 1980) and Frenkel et al. (Frenkel et al., 1983). Double emulsion also termed as emulsion of emulsion, are complex system, in which the droplets of dispersed phase themselves comprises even small of dispersed phase (Garti and Bisperink, 1998). Double emulsion (DE) droplets are mostly polydispersed in size. In some cases its droplets are big enough containing many small compartments with 50-100 droplets in each drop of double emulsion, while on the other hand small droplets of DE may consist of one or few droplets. There are two common types of DE; water-oil-water (w/o/w) and oil-water-oil (o/w/o). Two step processes are most commonly used for preparation of double emulsions, in which for w/o/w DE preparation, the inner aqueous phase (W_1) is dispersed in oil phase containing lipophilic emulsifier in the first step, which is followed by dispersion of the primary emulsion into outer aqueous phase (W_2) containing hydrophilic emulsifier (Garti and Bisperink, 1998; Schuch et al., 2013).

Florence et al (Florence and Whitehill, 1981b) identified that double emulsion droplets could be of three types (A, B, C), which did not exist exclusively in any one system but usually one of them is predominant. The type A, was found to be the simplest system consists of

relatively small droplets with almost single droplet of the internal aqueous phase (Fig.1 A). The droplet size in the type B emulsion system is larger composed of several small droplets (less than 50) of internal aqueous phase (Fig.1 B). The system became more complex (type C) when majority of droplets achieve relatively largest size, encapsulating numerous droplets of internal aqueous phase (W_1) (Fig.1 C). The system C, showed slow release of entrapped moiety than A or B, so system C would expected to be more promising in drug delivery system.

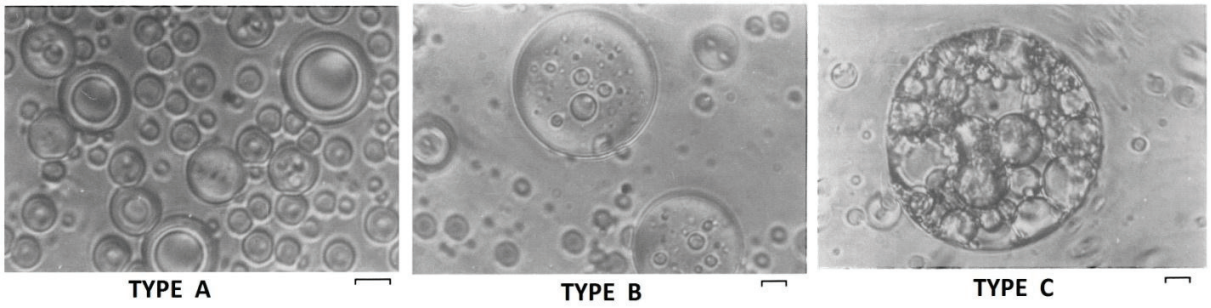

Fig. 1. Types of double emulsion droplets. (Florence and Whitehill, 1981b)

Double emulsion has the ability to prepare polymeric particles, which allow the controlled release of active ingredients soluble in the internal aqueous phase or dispersed in the polymeric matrix. Its foremost function is regarded as internal reservoir to entrapped active ingredients whatever you chose into the inner confined space, which can protect the entrapped sensitive ingredient against light, enzymatic degradation, and oxidation. It also allows the slow and sustained release of active ingredients from the internal reservoir to the external dispersion media and their key importance is due to their capability to encapsulate some water-soluble flavors and active ingredients(Benichou et al., 2004). In cosmetic field, it offers the possibility of combining incompatible ingredients in the same formulation to enhance the efficacy. Regarding disadvantages of double emulsion, it a complex process and thermodynamically unstable. The particles produced by this technique are comparatively heterogeneous and particle size is sensitive to various process parameter of double emulsion technique. Few prominent advantages and limitation of various techniques for encapsulation of drug are given in the table (Table 1). Single emulsion solvent evaporation, double emulsification, nanoprecipitation, emulsion diffusion and salting out techniques can be used to encapsulate lipophilic active agents. Double emulsion is a unique process encompassing the advantage of encapsulating both lipophilic and

hydrophilic drug molecules. Both emulsion diffusion and coacervation techniques are used for incorporation of thermosensitive drugs whereas phase inversion temperature method cannot be utilized to encapsulate thermolabile actives like peptides and proteins. Techniques that generally require high shearing stress and high pressure homogenization include single and double emulsification. Moreover, double emulsion generally gives polydisperse particles as compared to other techniques. Lastly, the techniques that do not require the use of toxic solvents or organic solvents are; emulsion diffusion, microemulsion, nanoprecipitation, high pressure homogenization and phase inversion temperature technique.

Table 1
Advantages and limitation of various techniques used for encapsulation of drug

Techniques	Advantages	Disadvantages	Examples
Single emulsion solvent evaporation	1. Provides high entrapment of lipophilic actives. 2. Size of particles is adjustable by changing homogenization speed, amount of stabilizer, viscosity of organic and aqueous phases.	1. Entrapment of hydrophilic drugs is poor. 2. It is difficult to scale up.	(Khalil et al., 2013) (Seremeta et al., 2013) (Guerreiro et al., 2012)
Double emulsion solvent evaporation	1. It provides an advantage of encapsulation of both hydrophilic and hydrophobic actives.	1. Large and non-uniform particles (polydisperse). 2. Two step process. 3. Leakage of the hydrophilic active into external aqueous phase. 4. Difficult to scale up.	(Zakeri-Milani et al., 2013) (Bitar et al., 2015) (Ibraheem et al., 2013)
Emulsion diffusion method	1. It allows incorporation of thermosensitive drugs. 2. Reduces mean particle size and narrow size distribution. 3. Good batch-batch reproducibility. 4. Higher entrapment of lipophilic drugs. 5. Use of nontoxic solvents. 6. Easy scale up process	1. Concentration of final formulation is required. 2. Possible organic solvent residues in the final formulation. 3. Poor encapsulation of hydrophilic drugs. 4. Longer time of emulsion agitation required. 5. Require larger volume of water for nanoparticles formation.	(Campos et al., 2013) (Hao et al., 2013) (Souguir et al., 2013)
Emulsion polymerization	1. It is fast and scalable.	1. Toxic organic solvents and monomers are used. 2. Difficult to remove residual monomers, initiators and surfactants from final product.	(Tolue et al., 2009) (Wu et al., 2011) (Sajeesh and Sharma, 2006)
Microemulsion Technique	1. Reduces mean particle size and narrow size distribution. 2. Organic solvent free method.	1. High concentration of surfactants and co-surfactants. 2. Concentration of final formulation is	(Zhang et al., 2006) (Destrée et al., 2007)

	3. No energy consuming process. 4. Easy to scale up.	required	
Nanoprecipitation	1. Simple and fast. 2. Uses non-highly toxic solvents. 3. High batch-batch reproducibility. 4. Does not require high shear stress. 5. Monodispersed particles.	1. Mostly limited to hydrophobic active encapsulation. 2. Nanoparticle size mainly depends on polymer concentration.	(Han et al., 2013) (Katara and Majumdar, 2013), (Siqueira-Moura et al., 2013) (Seremeta et al., 2013)
Salting out	1. No high shear stress is required. 2. Applicable to heat sensitive drugs. 3. High loading efficiency for lipophilic drugs. 4. Easy scale up. 5. High reproducibility.	1. Extensive nanoparticles washing step. 2. Exclusively applicable for only lipophilic drugs.	(Miller et al., 1988)
High Pressure Homogenization (Hot and Cold)	1. Good reproducibility. 2. Well established homogenization technology on large scale. 3. Organic solvent free method.	1. High temperature process. 2. High energy input 3. Complex equipment required. 4. Possible degradation of the components caused by high pressure homogenization.	(Shi et al., 2011) (Silva et al., 2011) (Kamiya et al., 2009) (Hoyer et al., 2010)
Coacervation method	1. Allows incorporation of thermosensitive drugs 2. Inexpensive for laboratory and industrial application. 3. Possibility to control shape and size of SLNs by reaction conditions.	1. Possible degradation of the components under acidic conditions.	(Wieland-Berghausen et al., 2002)
Phase Inversion Temperature Method	1. Organic solvent free method. 2. Non-energy consuming method. 3. Easy to scale up.	1. Not suitable for thermosensitive molecules like peptides or proteins.	(Alvarado et al., 2013) (Saberi and McClements, 2015)
Solvent Injection Method	1. Easy to handle. 2. Fast production.	1. Possible organic solvent residues in the final formulation.	(Schubert and Müller-Goymann, 2003) (Arica Yegin et al., 2006)

3. Formulated double emulsion based dispersion

The most often used double emulsion technique for preparation of miro- and nanoparticle, and encapsulation of active molecules is double emulsion solvent evaporation technique. Initially, this technique was used for microencapsulation (Alex and Bodmeier, 1990; Pisani et al., 2008). In this method, homogenization is performed in two steps; in the first step, water soluble drugs are incorporated in the inner aqueous phase (W1) and polymer/ lipophilic drugs are added into oil phase (O), then both phases are homogenize by proper agitation to form the primary emulsion (W1/O). Then, the primary emulsion is emulsified with the outer aqueous phase containing appropriate stabilizer to form double emulsion (W1/O/W2). Formation of double emulsion (particulate dispersion) is followed by evaporation of the organic solvent (O) from the dispersed phase leading to a point of insolubility and consequently, hardening of the polymer encapsulating the active material. The solvent may be evaporated under reduced pressure via rotary evaporator or by simple stirring at ambient temperature depending upon the boiling point of organic solvent. The outer aqueous phase act as dispersion medium and the agitation can be provided either by mechanical stirring or sonication depending upon the nature of drug to be encapsulated and the intended particle size.

3.1. Commonly used polymers in double emulsion solvent elimination preparations

Biodegradable polymers have been used tremendously in drug delivery system for encapsulation of pharmaceuticals and pharmaceuticals as they can be degraded into nontoxic components. These polymers are consisting of ester, amide and ether functional groups. Different polymers such as PLA, PLGA and PCL are being intensively researched for encapsulation various active drugs, genes and macromolecules using double emulsion process (Badri et al., 2014; Pillai and Panchagnula, 2001; Rosset et al., 2012). PCL is a nontoxic, biodegradable and biocompatible polymer; with low glass transition temperature and melting point and its metabolites are eliminated from the body by innate metabolic process. It has been extensively study for encapsulation via double emulsion process for control drug delivery system in several formulations due to its compatibility with wide range of drugs and its slow degradation to release drug for extended period of time (Florindo et al., 2008a; Wang et al., 2008a) (Iqbal et al., 2014; Lowery et al., 2010). Poly(lactic-co-glycolic acid) is another biocompatible and biodegradable polymers commonly used in double emulsion techniques (Hattrem et al., 2014; Rizkalla et al., 2006; Takai et al., 2011). After its approval by FDA for use in humans, it has

been a popular choice for drug delivery. It degrades gradually into the biocompatible products of lactic and glycolic acid via hydrolysis and thus releases the encapsulated agents slowly over a long period of time (weeks-months). This polymer is available in different PLGA/PLA ratio. As lactic acid is more hydrophobic than glycolic acid and, thus, lactic acid-rich PLGA copolymers are less hydrophilic, and degrade very slowly (Dinarvand et al., 2011). PLGA has been investigated for encapsulation of protein, peptides and nucleic acids, and for attachments of legends to targeting particle to specific tissues and imaging (McCall and Sirianni, 2013).

Poly (L-lactic acid) (PLA) polymers are approved by FDA in humans (Zille et al., 2010) and have been frequently used for encapsulation in drug delivery system via double emulsion process (Hong et al., 2005; Montiel-Eulefi et al., 2014; Nihant et al., 1994). It belongs to the most promising category of biodegradable polymers having excellent mechanical properties, good biocompatibility, and low toxicity. It is semi-crystalline polymer with a melting point of about 180 °C, glass transition temperature of about 55 °C and tensile strength of 50–70 MPa and commonly prepared by ring-opening polymerization (ROP) (A. Auras et al., 2010; Södergård and Stolt, 2002) . The properties such as crystallinity, hydrophobicity and melting point of PLA can be tailor made by copolymerization (random, block, and graft) with other comonomers, modification in molecular architecture (hyperbranched, star shaped, or dendrimers), functionalization or blending with other polymers. For example, glycolide, ε-caprolactone, δ-valerolactone, trimethylene and carbonate have been often used to modify its thermal properties (GRUVEGÅRD et al., 1998). These modifications also affect drug loaded PLA particles properties such as drug release rate, permeability and degradation rate of the matrix. Beside many advantages of biodegradable PLA polymers in controlled drug delivery systems, they have certain shortcomings such as a risk of toxicity and immunogenicity due to their acidic by-products when used for long period of time (Naraharisetti et al., 2005).

The selection of the appropriate polymer during encapsulation of drug is a critical step, which depends upon the chemical nature of the drug and polymer and their intended application. Table 2 is listing some of the most commonly used biodegradable polymers in encapsulation via double emulsion process.

3.2. Stabilizers

Stabilizers make it possible to maintain the physicochemical state of a dispersion of two or more immiscible phases and prevent the separation of phases, thus making emulsion system

more stable. The commonly used stabilizers in double emulsion process include PVA, Tween 80 and Span 80. However, poly vinyl alcohol (PVA) is one of the most frequently used stabilizers in double emulsion process for encapsulation of different active moieties (Liu et al., 2005a; Rizkalla et al., 2006; Yang et al., 2001). It is a well-known hydrophilic, biocompatible polymer and possesses good mechanical strength, low fouling potential, and lasting temperature stability and pH stability. These properties of PVA make it suitable candidate to be used in encapsulation of various pharmaceuticals and biopharmaceuticals (Xia and Xiao, 2012).

Table 2
Examples of polymers, stabilizers and solvents used in encapsulation of various actives/nonactives via double emulsion method

Polymer used	Active molecules /solid ingredient	Organic solvent	Stabil-izers /surfa-ctants	use/objective/Purpose	Avera-ge size	References
PLGA	Clonidine (hydrophilic molecule)	Methylene chloride	PVA	Sustained release for intra-articular administration	10-20 μm	(Gaignaux et al., 2012)
PLGA	DNA	DCM	PVA	Parameters optimization (PLA nanoparticles exhibit high release of DNA)	100-300nm	(Rizkalla et al., 2006)
PLGA	Hydroxyapatite	DCM	PVA	System optimization	-	(Takai et al., 2011)
PLGA	SiO_2	Chloroform	PVA	System optimization	-	(Takai et al., 2011)
PLGA	Tartrazine (synthetic dye)	Corn oil	Tween 80	Tartrazine encapsulation and release	15-25μm	(Hattrem et al., 2014)
PLGA	BSA (bovine serum albumin)	Chloroform	PVA	Residual PVA effect on cellular uptake of nanoparticle	380-520nm	(Sahoo et al., 2002)
PLGA	Vancomycin	Methylene chloride	PVA	Improving intestinal permeability of vancomycin administered via orally	450-466 nm	(Zakeri-Milani et al., 2013)
PLGA	Alendinate	Ethyl acetate	PVA	Loading efficiency evaluation	145-223 nm	(Cohen-Sela et al., 2009)
PLGA	MEP421 (peptide) & BSA	DCM-ethyl acetate (3/2, v/v)	PVA	MEP421 (for Alzheimer's disease therapy) encapsulation for sustained drug release	21-25 μm	(Ji et al., 2008)
PLGA	SPf66 (vaccine for preventing malaria)	Methylene chloride	PVA	Evaluation of the effect of γ-irradiation on PLGA microspheres containing SPf66.	1.6-2 μm	(Igartua et al., 2008)
PLGA	Pingyangmycin (anti-tumor antibiotic)	DCM	PVA	Pingyangmycin encapsulation for sustainably release	300 nm-10 μm	(Han et al., 2010)
PLGA	BSA	DCM	PVA	Protein loaded polymeric	3-7 μm	(Ravi et al., 2008)

				microspheres development for controlled release of drug		
PLGA	Interferon-alpha (IFN-α) 2b	DCM	PVA	Sustain drug delivery	45-110 µm	(Zhang et al., 2008)
PLGA	Egg-white lysozyme	Methylene chloride	PVA	Preservation of lysozyme via encapsulation	-	(Pérez et al., 2002)
PLGA	Ciprofloxacin HCl	DCM	PVA	Ciprofloxacin antibacterial potential evaluation	130-353 nm	(Jeong et al., 2008)
PLGA	Proteins (tetanus toxoid, lysozyme, and insulin)	Ethyl acetate and methylene chloride	PVA	Process-related stability issues evaluation in encapsulation of proteins	353-468 nm	(Bilati et al., 2005)
PLA	BSA (bovine serum albumin)	Methylene chloride	PVA	Controlled release devices	98-121µm	(Yang et al., 2001)
PLA	Oligonucleotide (DNA)	Methylene chloride	PVA	Preparation of porous via leaching of pore former (glycerol monooleate), and loading with oligonucleotide for antisense therapy	80-220 %m	(Ahmed and Bodmeier, 2009)
PLA	rhi (recombinant human insulin)	DCM+ Toluene	PVA	Parameters optimization	16 µm	(Liu et al., 2005b)
PLA	Recombinant human epidermal growth factor (rhEGF)	Methylene chloride	PVA	Encapsulation of rhEGF(peptide) for chronic gastric ulcer healing.	75-130 µm	(Han et al., 2001)
PLA	HSA	Methylene chloride	PVA	Parameters optimization	200nm	(Zambaux et al., 1998)
PLA	-	Propylene carbonate, DCM	PVA	Particles formation mechanism	169-188 nm	(Quintanar-Guerrero et al., 1997)
PLA	Ovalbumin	DCM	PVP/ NaCl	Proteins encapsulation mechanism	14 µm	(Chen et al., 2002)
PLA	Acridine orange (fluorescent dye)	Ethyl acetate	Pluro-nic f68	Particles Transition behavior study	500-1000 nm	(Ji et al., 2012b)

PLA	BSA (bovine serum albumin)	Methylene chloride	Tween 80	Nanoparticle preparation without stabilizer	200-500 nm	(Lu et al., 1999)
PLA	-	Propylene carbonate	PVA: polysor-bate 80	Process optimization	150-450 nm	(Quintanar-Guerrero et al., 1996)
PLA	Insulin	DCM	Tween 80	Influence of surfactant on Insulin encapsulation (an antidiabetic agent)	169-253 nm	(Zhu et al., 2005)
PCL	Vancomycin (antibiotic)	Methylene chloride	-	Ophthalmic controlled drug delivery	-	(Petitti et al., 2009)
PCL	Disodium norcantharidate	DCM	PVA	Encapsulation of anticancer drug	39-181 µm	(Wang et al., 2008b)
PCL	Streptococcus equi antigens	DCM	PVA	Vaccine adjuvant, to protect animals against strangles	1.43-2.35 µm	(Florindo et al., 2008b)
PCL	Miglyol 812 (oil)	Ethyl acetate	PVA	Particles formation mechanism	450 nm	(Moinard-Chécot et al., 2008)
PCL	Insulin	DCM	PVA	Insulin encapsulation	0.75-30 µm	(Mukerjee et al., 2007)
PCL	-	DCM	PVA	Process parameters optimization	2-8 µM	(Ibraheem et al., 2014a)
Eudragit	Propranolol HCl (hydrophilic), Nife-dipine (lipophilic)	Methylene chloride	PVA	2 drug encapsulation in non-degradable microparticles for controlled release	85-130 µm	(Hombreiro-Pérez et al., 2003)
Eudragit S100	Theophylline	DCM	Spane 80	Drug encapsulation.	122-339 µm	(Lee et al., 2000)
Eudragit S100	Enalapril Maleate	DCM: ethanol: Isopropyl alcohol, (5: 6: 4)	Tween 80	developed for hypertension and congestive heart failure, was used as a model peptide	9-18 µm	(Rawat Sing et al., 2011)
PLA-PEG	Plasmid DNA	Ethyl acetate: Methylene	PVA	Plasmid DNA encapsulation to avoid its degradation in the body, it has application in therapy and	272-296 nm	(Perez et al., 2001)

		chloride (1:1)		vaccine.		
PLA-PEG-PLA	Insulin	Acetone	Tween 80, dextran T-70	Insulin encapsulation (an antidiabetic agent)	250nm	(Ma et al., 2001)
PLGA-PCL	BSA	Methylene chloride	PVA	Process parameter optimization	200-900 nm	(Lamprecht et al., 2000)
PLA or PLGA	Melittin (peptide)	DCM	PVA	Development of controlled release injection to deliver the melittin over a month.	5 µm	(Cui et al., 2005)
PLGA/PCL blend (ratio:80:20; 60:40; 40:60)	Doxycycline	DCM	PVA	controlled delivery of doxycycline in the treatment of human periodontal pocket	90-200 µm	(Mundargi et al., 2007)
poly methacr-ylic Acid	Doxorubicin	Chloroform	PVA	Co-delivery of Anti-cancer drugs	174 nm	(Chiang et al., 2014)
polybutyl adipate	Penicillin-G	DCM	Tween	Investigation of Surfactants role on particle size of nanocapsules containing penicillin-G	131-247 nm	(Khoee and Yaghoobian, 2009)

PLGA=poly(lactic-co-glycolic acid), PLA= poly lactic acid , DCM= dichloromethane, PVA= polyvinyl alcohol, HAS= human serum albumin, PLA-PEG= = poly(lactic acid) -poly (ethylene glycol). BSA=bovine serum albumin, HSA =Human serum albumin

4. Encapsulation of active ingredients

The selection of a specific technique for an efficient drug encapsulation is generally determined by the hydrophilicity or hydrophobicity of the drug molecules (Jelvehgari and Montazam, 2012). Despite the fact that various techniques have been described in the literature as well as successfully employed by researchers to encapsulate hydrophobic agents into biodegradable nanoparticles (Ibrahim et al., 2013; Palamoor and Jablonski, 2013, 2014), the encapsulation of hydrophilic drugs into such carriers is not facile. This is because the hydrophilic compound is pushed out from hydrophobic matrix into the dispersing water phase during formulation of the particles (Ibrahim et al., 2013). W/O or O/O emulsion prepared via solvent evaporation methods are quite suitable for biologically active substances encapsulation into microspheres but certain problems are involved in these techniques e.g. it is difficult to remove the large volumes of solvents from the dispersions (Jelvehgari and Montazam, 2012). The mineral or vegetable oil used as external phase in both w/o or o/o emulsion makes washing/collecting of resultant particles difficult. However, more innovative method to encapsulate hydrophilic drugs via emulsion solvent evaporation technique includes double emulsions (multiple emulsions) e.g. in w/o/o emulsion type, the problems associated with w/o or o/o methods are eliminated. Jelvehgari and Montazam (Jelvehgari and Montazam, 2012) prepared theophylline loaded microparticles via emulsion-solvent extraction or evaporation techniques to compare the results (Fig. 2). In any case, the formulation should be adapted to the chemical nature of used polymer and the chemical stability of the active molecule in the used solvent and encapsulation conditions.

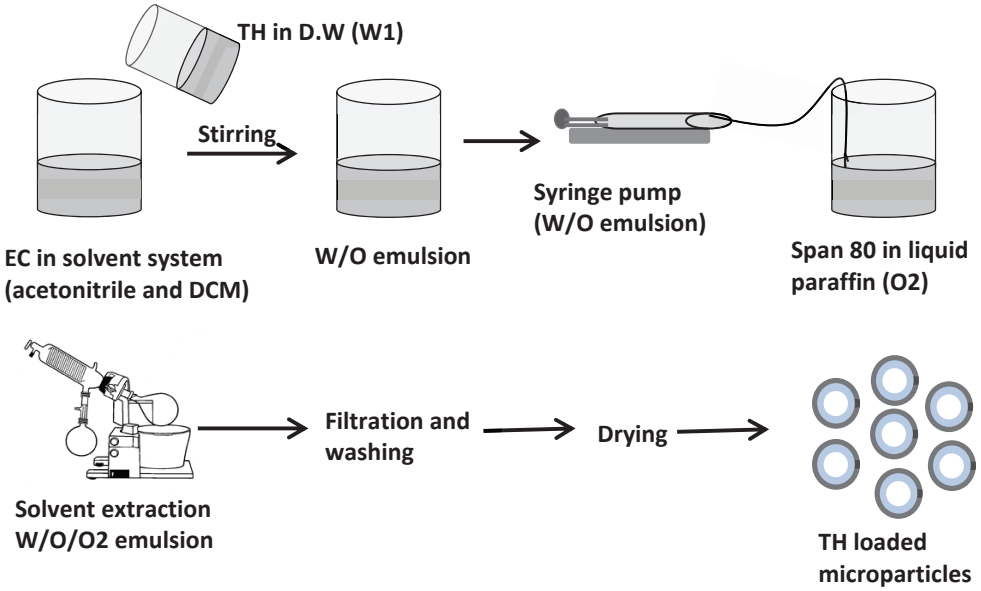

Fig. 2. Ethyl cellulose loaded microparticles prepared via double emulsification ($W/O_1/O_2$) technique (Jelvehgari and Montazam, 2012).

4.1. Encapsulation of common pharmaceutical molecules by double emulsion solvent evaporation process

Some common pharmaceutical drugs encapsulated into particles by double emulsion process such as anticancer drugs, anti-inflammatory and antibiotics are reviewed here. The most encapsulated active molecules are anticancer drugs.

4.1. 1. Anticancer drugs

Among anticancer drugs, doxorubicin, cisplatin, fluorouracil and epirubicin have been encapsulated by this technique (Ji et al., 2012a; Matsumoto et al., 1997; Wang et al., 2010; Zhou et al., 2006). Doxorubicin is an antitumor drug and has been frequently incorporated into different polymeric particles prepared by double emulsion process (Amjadi et al., 2013; Jiang et al., 2011). It is an antibiotic commercially available as a water soluble hydrochloride salt, which used in cancer chemotherapy. Tewes et al (Tewes et al., 2007) prepared PLGA particles loaded with doxorubicin. The hydrodynamic size of DOX loaded particles was found to be 316 nm and its encapsulation efficiency was 67%. The release rate was slow and over a period of 24 hours only 1.5% of drug was released, which indicates the controlled release of formulation. Release rate depends upon several factors such as water-solubility, dissolution, particles size and

thickness of polymeric coating (Pearnchob et al., 2003; Siepmann et al., 2004).In another study, Wang et al (Wang et al., 2011) demonstrated that the anti-tumor efficacy of doxorubicin (DOX) and paclitaxel (TAX) can be enhanced by its co-delivery strategy. They prepared drug-loaded Methoxy poly(ethylene glycol)-poly(lactide-co-glycolide) (mPEG-PLGA) nanoparticles by double emulsion process. The hydrophilic doxorubicin was incorporated in inner aqueous phase and the hydrophobic paclitaxel in methylene chloride (oil phase) and emulsified by sanitation. Average size of drug loaded particles (NPs-DOX-TAX) was measured by DLS and found to be 243 nm. The in vitro drug release profile was determined in phosphate-buffered saline (PBS) at pH 7.4 and pH 4.4 and the release rate of both DOX and TAX were pH dependent (Fig. 3). At neutral pH, the release rates of both drugs were slower than acidic pH, and about 90% of drugs were released within 300 hours at pH 7.4.

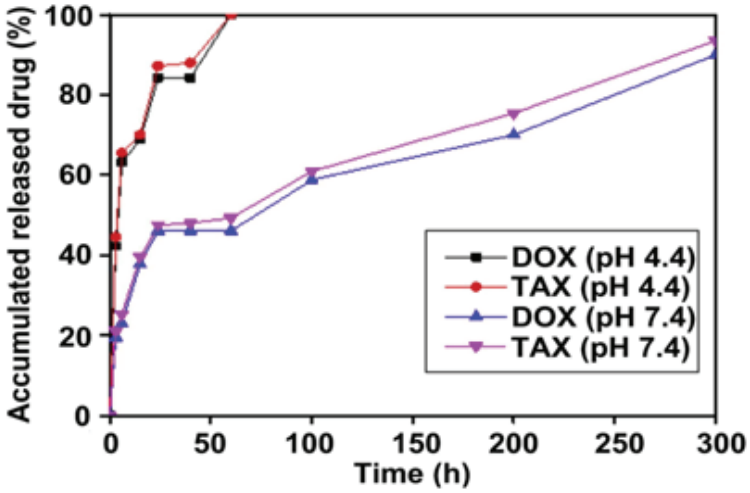

Fig. 3. Release profiles of DOX and TAX from mPEG-PLGA nanoparticles in PBS at 37 °C at pH 4.4 (black, red) and pH 7.4 (blue, purple) (Wang et al., 2011).

4.1. 2. Anti-inflammatory drugs

Anti-inflammatory drugs encapsulated by double emulsion process included acetaminophen, aceclofenac , diclofenac sodium, aspirin and ketoprofen etc (Bhatnagar et al., 1995; Lai and Tsiang, 2005; Nagda et al., 2009; Pavanetto et al., 1996). Aspirin is widely utilized nonsteroidal anti-inflammatory drug (NSAID). It may be used to reduce pain and swelling in conditions such as arthritis, to reduce fever and relieve mild to moderate pain from conditions such as muscle aches, toothaches, common cold. Fargnoli et al (Fargnoli et al., 2014)

encapsulated aspirin and prednisolone drugs in poly-lactic glycolic acid polymer (PLGA) nanoparticles by double emulsion process for cardiac gene therapy. And it was found that aspirin particles have large size (323 nm) than prednisolone nanoparticles (234 nm), this difference in size was attributable to higher aspirin mass content. Loading Efficiency results were uniform for all nanoparticle types, i.e. PLGA 50:50 Prednisolone [88.9 %], PLGA 65:35 Prednisolone [88.2 %], PLGA 50:50 Aspirin [89 %] and PLGA 65:35 Aspirin [88.8 %]. The control release of drug for over a time period of 5 days are shown in Fig. 4. The release rate of aspirin was faster as compared to prednisolone as shown in the Fig. 4.

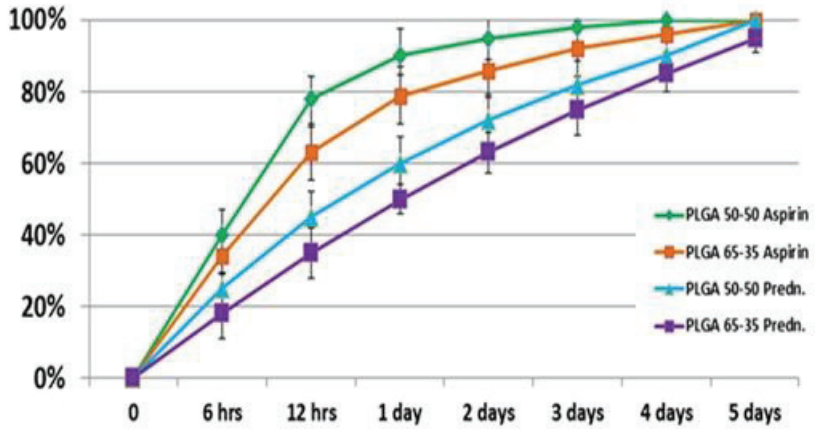

Fig. 4. PLGA Nanoparticle formulations release analysis. Controlled release study results demonstrate that aspirin particles overall release faster than prednisolone types (Fargnoli et al., 2014).

4.1. 3. Antibiotic drugs

Antibiotics such as, erythromycin, gentamicin, norfloxacin, cephalexin, capreomycin (Chaisri et al., 2009; Fan et al., 2009; Huang and Chung, 2001) Cefazolin, ciprofloxacin, clindamycin, colistin, doxycycline,and vancomycin (Shah et al., 2014) have been encapsulated by double emulsion technique. Encapsulation of highly hydrophilic drugs such as gentamicin has been performed by many researches via double emulsion method (Virto et al., 2007; Yang et al., 2001). Gentamicin is the most important aminoglycoside that has been used widely for the treatment of osteomyelitis and against a wide range of Gram-positive and Gram-negative bacteria (Lecároz et al., 2006). However, a prolonged *dosage regimen of antibiotics* (4–6 weeks) for the treatment of osteomyelitis is needed, which may cause systemic toxicity. Thus, localized

drug delivery of gentamicin has been proposed, in order to minimize the side effects by reducing administered dose and to reduce probability of drug resistance (Huang and Chung, 2001). The in vitro drug release is dependent on the distribution of drug within microsphere, polymer blend ratio, polymer degradation pattern, stabilizer and size of microsphere (Faisant et al., 2002; Lecomte et al., 2005; Luan et al., 2006, 2006). The high initial burst release of drug is often attributed to the associated drug on the particles surfaces (Kassab et al., 1997). On the other hand, larger size particle release drug more slowly and for extended period of time compared to smaller particles (Berkland et al., 2003).

In a study conducted by Shah et al (Shah et al., 2014) cefazolin, ciprofloxacin, clindamycin, colistin, doxycycline and vancomycin were loaded at 10 wt% and 20 wt% into PLGA microparticles using double emulsion solvent evaporation technique. All the antibiotics were first solubilized in the inner aqueous phase at 37 C° and then added to the oil phase (PLGA dissolved in methylene chloride) at a ratio of 1:5.6 v/v. The internal phase/oil phase emulsion was then homogenized with outer aqueous phase (0.3 wt% PVA solution) at 700 rpm. Finally, the solvent was evaporated and the particles were washed. Table 3 shows their loading efficiencies.

Table 3
Loading Efficiencies of Microparticle Formulations (Shah et al., 2014)

	Loading efficiency of 10 wt% antibiotic-loaded PLGA MPs (%)	Loading efficiency of 20 wt% antibiotic-loaded PLGA MPs (%)
Cefazolin	36.4±3.3	51.3±6.1
Ciprofloxacin	86.8±10.9*	38.9±17.8*
Clindamycin	84.9±6.9	89.5±4.0
Colistin	102.0±5.0	105.3±4.4
Doxycycline	71.4±2.3	89.4±0.9
Vancomycin	83.3±4.8	76.9±6.2

Data is presented as mean ± standard deviation, n = 3 per group

* Indicates significant difference between 10 and 20 wt% loaded for a given antibiotic

The release kinetics of all of the antibiotics loaded PLGA particles Were determined in phosphate buffered saline at pH 7.4. The in vitro release curves are shown in Fig. 5. From the release analysis, it was found that during phase-1 (0-1 day), 20 wt % loaded ciprofloxacin formulation has greater burst release as compared to 20 wt % clindamycin, cefazolin and

doxycycline loaded formulations, while no significant difference was found between 20 wt% cefazolin, clindamycin and doxycycline formulations. Most of the loaded drugs were released during phase-2 (1-21 days). In this phase, drug release rates were almost same for cefazolin, ciprofloxacin, clindamycin, and doxycycline for both 10 wt % and 20 wt % loaded particles. In case of colistin and vancomycin loaded particles, the drugs were mainly released during phase 3 (24-38 days) and there was no significant difference in release rates between colistin and vancomycin loaded particles (Shah et al., 2014).

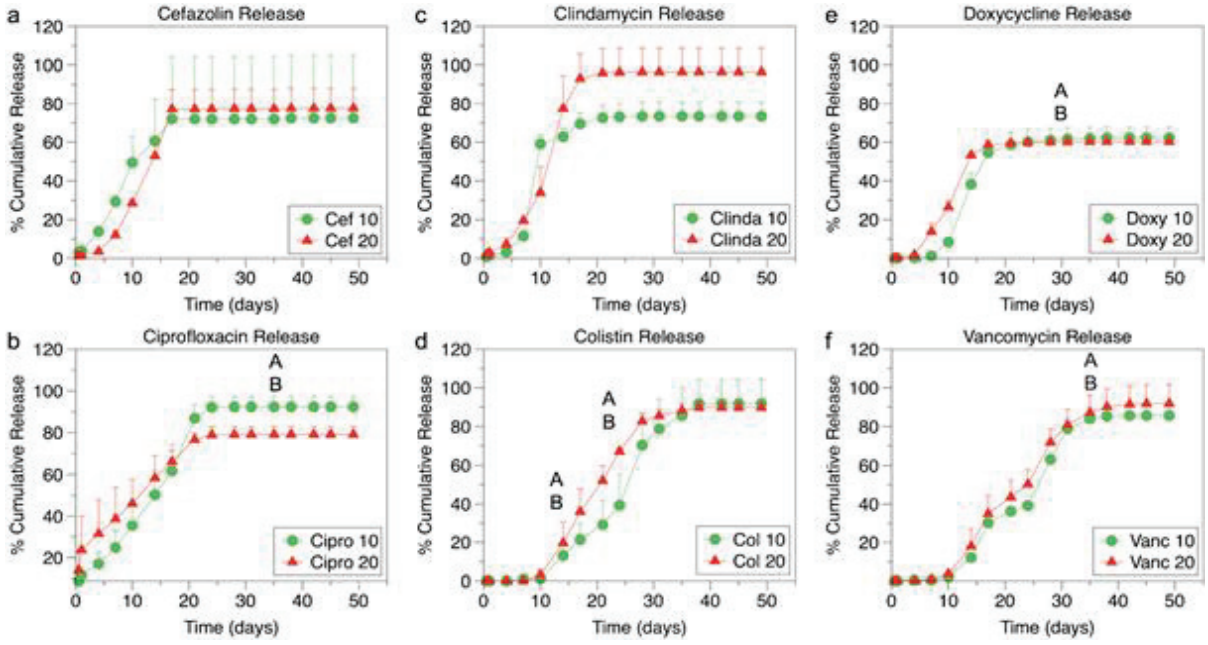

Fig. 5. Release curves for (a) cefazolin, (b) ciprofloxacin, (c) clindamycin, (d) colistin, (e) doxycycline, and (f) vancomycin loaded PLGA microparticles at 10 and 20 wt%. Significant differences between release rates during each phase for the same antibiotic are indicated by letters A and B. For colistin loaded PLGA MPs, Phase 2 and Phase 3 are significantly different between 10 and 20 wt% loaded MPs ($p<0.05$). Ciprofloxacin and doxycycline 10 wt% loaded MPs demonstrate increased release of antibiotic during Phase 3 compared to 20 wt% loaded MPs, and vancomycin loaded MPs demonstrate increased release from 20 wt% MPs compared to 10 wt% MPs in phase 4 ($p<0.05$). Cumulative percent release is the same between 10 and 20 wt% for any antibiotic ($p>0.05$). The three release phases of cefazolin, ciprofloxacin, clindamycin and doxycycline are: phase 1 (0-1d), phase 2 (1-21d), phase 3 (21-49d), and four phases of colistin and vancomycin are: phase 1 (0-10d), phase 2 (10-24d), phase 3 (24-38d) and phase 4 (38-49d) (Shah et al., 2014).

4.2. Encapsulation of biopharmaceuticals via double emulsion method

4.2. 1. Encapsulation of proteins

Advancement in the field of biotechnology/genetic engineering (Hutchinson and Furr, 1990) and better understanding of the role of peptides and proteins in the physiology and pathology has enhanced the importance of peptides and proteins as therapeutic agents. However, certain hurdles are associated with the therapeutic usage of the available peptides and proteins such as their short half-life. Moreover, these can be easily degraded and most peptides possess poor passage through biological barriers due to their poor diffusivity and low partition coefficient (Lee, 1988). Due to these reasons, during the past two decades, the researchers have focused their interest in encapsulation of these agents within colloidal particles by employing different biodegradable polymers (Arshady, 1991; Heller, 1993; Jalil and Nixon, 1990; Langer, 1990; Zhou and Wan Po, 1991). Encapsulation protects these agents from degradation, controls their release from site of administration and in some cases, it can also improve the passage through biological barriers. Although the double emulsion encapsulation technique is taken as a complex process, it is still widely employed to encapsulate hydrophilic agents, particularly protein and peptide drugs, into polymeric microspheres resulting in higher encapsulation efficiencies (Alonso et al., 1994; Bley et al., 2009; Kreitz et al., 1997; Leach and Mathiowitz, 1998). Engel et al. (Engel et al., 1968) successfully encapsulated insulin via this technique to enhance the efficiency of insulin upon oral administration as well as to facilitate its gastrointestinal absorption.

Protein encapsulation via double emulsion is performed in two steps (Nihant et al., 1994; Tan and Danquah, 2012): In the primary emulsion formation stage, the aqueous solution of protein is added to the polymeric organic solution in the presence of high shearing forces (ultrasonication/homogenization). In the second stage, double emulsion (w/o/w) is formed by dispersing the primary emulsion in an external aqueous phase containing suitably selected stabilizer. Finally, organic solvent removal either by evaporation or extraction results in the formation of protein loaded particles (Fig. 6).

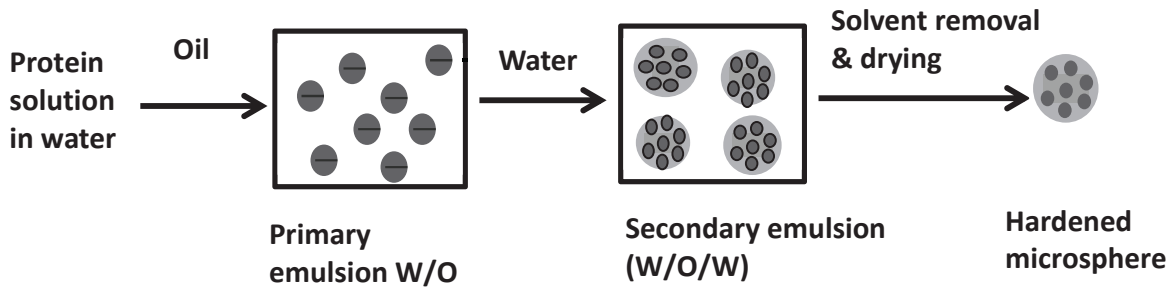

Fig. 6. Double emulsion solvent evaporation method for microencapsulation of proteins (Yeo et al., 2001).

Many proteins have been encapsulated via this technique such as bovine serum albumin (Benoit et al., 1999; Lu et al., 1999; Sah et al., 1995; S. R. Jameela, 1996; Yang et al., 2001; Youan et al., 1999). Lamprecht et al. (Lamprecht et al., 2000) also prepared BSA based nanoparticles via double emulsion pressure homogenization technique with the goal to investigate the influence of miscellaneous process control parameters on the obtained nanoparticles. Some novels method for the encapsulation of proteins and peptides were also introduced such as Viswanathan et al. (Viswanathan et al., 1999) introduced a (water in oil) in oil emulsion. They used oil as processing medium to prevent the hydrophilic proteins from diffusing out of the microspheres before they harden. Whereas, in another case, BSA loaded PCL microparticles were prepared by Lin and Huang, (Lin and Huang, 2001a) via w/o/o/o emulsion solvent evaporation method. They utilized two types of homogenizers and investigated the influence of solvent evaporation rate on the crystallinity and performance of particles. Another study was also performed on similarly prepared particles i.e. via w/o/o/o emulsion technique (Lin and Huang, 2001b) to study the effect of pluronics on the BSA loaded microparticles.

A luteinizing hormone-releasing hormone (LHRH) agonist i.e. Leuprolide acetate was encapsulated via w/o/w emulsification by Ogawa et al. (Ogawa et al., 1988a) and Okada et al. (Ogawa et al., 1988b). Currently this product is available in the market. There are many other examples of proteins encapsulated by double emulsion technique and few of these examples are listed in the following table (Table. 4).

Table 4
Various proteins encapsulated via double emulsion technique using different polymers

Protein	Polymer	Encapsulation efficiency	Initial release in 1 day	Reference
Carbonic anhydrase	PLGA	45-48%	< 10%	(Lu and Park, 1995)
BSA	PLGA	56-85%	30-50%	(Igartua et al., 1997)
Recombinant human growth hormone (rhGH)	PLGA	40-66%	20%	(Cleland et al., 1997)
Urease	PLGA	17-55%	17-45%	(Sturesson and Carlfors, 2000)
Leuprolide	PLGA	13.4%	10%	(Woo et al., 2001)
Brain derived neurotr-ophic factor (BDNF)	PLGA,PLL, PEG	-	BDNF delivered for longer than 60 days	(Bertram et al., 2010)
Vascular endothelial growth factor (VEGF)	PLGA	46-60%	-4ng/mL -27ng/mL	(Karal-Yılmaz et al., 2011)
Alpha-1 antitrypsin (α1AT)	PLGA	Varies with copolymer ratio of PLGA	-	(Pirooznia et al., 2012)
Lysozyme	PEG/PBT block	89%	< 5%	(J M Bezemer, 2000)

Although, this technique is widely used for protein encapsulation, still there are certain limitations associated with this method. The drug encapsulation efficiency is not very good and the production cost of protein drugs is high. Moreover, the use of toxic organic solvents such as dichloromethane and ethyl acetate is also not very favorable. Furthermore, protein drugs tend to denature and form aggregates due to various factors like high shearing forces and exposure to large interface between aqueous and organic phases.

4.2. 2. Encapsulation of Nucleic acids

Nucleic acid is an important biomolecule in our body having many applications in gene therapy and diagnostics (Chen et al., 2009). However the major challenge is its effective and efficient delivery to the site of interest without its degradation. Different strategies have been adopted for this purpose such as, complexation of DNA with polycations (Möbus et al., 2012; Putnam, 2006) encapsulation within liposomes (Edwards and Baeumner, 2007; Tsumoto et al.,

2001), encapsulation via double emulsion (Ibraheem et al., 2013a) and polyelectrolyte capsules (Kreft et al., 2006; Shchukin et al., 2004). In modern drug delivery system, it is a challenge to administered hydrophilic biomolecules such as nucleic acids. Because of their water solubility, they tend to diffuse into continuous aqueous phase during emulsification process (Iqbal and Akhtar, 2013; Luo et al., 1999; Perez et al., 2001; Woodrow et al., 2009) which leads to low encapsulation efficiency of active moiety (Cun et al., 2011). Additionally, the negative charged biomolecules are unable to penetrate the cell membrane appropriately and also labile to degradation by different enzymes such as proteases and nucleases (Amidon et al., 1995; Li et al., 2006; Verdine and Walensky, 2007).In order to overcome these problems, researchers have focused on encapsulation of nucleic acid drugs inside biodegradable polymer, because it protecting the nucleic acid against degradation, facilitating its intracellular penetration, minimizing fluctuation in plasma concentration, enhancing the drug delivery to intended site and controlling the rate of drug release by diffusion (Lecomte et al., 2004; Singh et al., 2010; Streubel et al., 2006)

Various approaches have been used for encapsulation of nucleic acids including emulsion solvent evaporation, coacervation, spray drying and double emulsion techniques (Oster and Kissel, 2005; Tan and Danquah, 2012; Zhao et al., 2014). However double emulsion is technique is considered one of the most appropriate method, especially for encapsulation of hydrophilic nucleic acid drug inside biodegradable polymer (Jeffery et al., 1991, 1993; Mehta et al., 1996), this technique is reproducible and can be scale up for large batches (Jorgensen and Nielson, 2009). Typically in this method, the aqueous solution of nucleic acid is mixed with a solution of polymer dissolved in organic solvent by using sonication or homogenization techniques (Ducheyne et al., 2011). Beside several advantages, double emulsion process has a drawback of shear force used for homogenization of nucleic acid's solution in the organic phase. Which can damage the integrity of biomolecules, thus leads to loss of its biological activity. Moreover, the organic solvent can adversely affect the structure of nucleic acid during homogenization. This damage can be minimized by condensation of nucleic acid with cationic polymers, in order to reduce its size and maintain its supercoiling structure, and thus preserve its biological activity (Ducheyne et al., 2011). It has been established that during second step of double emulsion emulsification, the applied shearing force disrupts the primary emulsion droplets, thus the inner aqueous phase containing nucleic acid mixed with outer aqueous phase, allowing diffusion of

nucleic acid into outer aqueous phase. Which may leads to poor loading efficiency, in systems with low stability of primary emulsions (Jorgensen and Nielson, 2009). The ratio of aqueous phase to oil phase has a significant effect on double emulsion stability. It has been reported that, an emulsion with ratio of 1:10 (water: oil) is more stable than ratio of 1:5 (water: oil) (Mohamed and van der Walle, 2006). Similarly, the emulsion of smaller particle size can be prepared with water: oil ratio of 1:30 as compared to ratio of 1:10 (water: oil), however beyond water: oil ratio of 1:20 a decrease in encapsulation efficiency has also been observed, thus water: oil ratio in between 1:10 and 1: 20 are frequently used (Hsu et al., 1999).

For encapsulation of nucleic acid, both homogenization and sonication process can be used, which results in almost same % encapsulation efficiency. Though, homogenization is the most commonly used technique. In case of sonication technique, there are concerns regarding scale up of process to produce large batches and stability of larger biomolecules (Hsu et al., 1999). The particle size decreases with an increase in homogenization speed (Díez and Tros de Ilarduya, 2006). However, this is not ideal choice to decrease the particle size, because vigorous homogenization in second step of emulsification can disrupt the primary emulsion droplet and consequently, leakage of nucleic acid into the outer aqueous phase may occur (Jorgensen and Nielson, 2009). Encapsulation efficiency of nucleic acid can be further enhanced by addition calcium phosphate to inner aqueous phase of double emulsion. Dördelmann et al (Dördelmann et al., 2014) reported that, by addition of calcium phosphate to inner aqueous phase of double emulsion, the encapsulation efficiency of siRNA and DNA was increase by 37% and 52% respectively, compared to nanoparticle without calcium phosphate (Fig. 7). They obtained nucleic acid loaded PLGA nanoparticles with a diameter of 200 nm and zeta potential of -26 mV, prepared by a double emulsion solvent evaporation technique and stabilized with PVA. The addition of polyethyleneimine has changed the zeta potential to +30 mV, thus facilitating the cellular uptake of these particles.

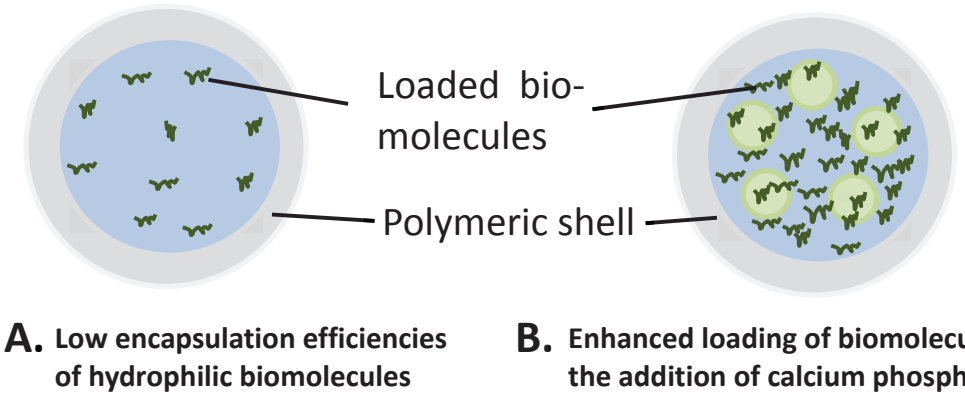

Fig. 7. Schematic illustration of the improved loading efficiency of nucleic acids by the addition of calcium phosphate nanoparticles. (A) PLGA nanoparticles, (B) Calcium phosphate-PLGA nanoparticles (Dördelmann et al., 2014)

4.2. 3. Encapsulation of miscellaneous biopharmaceuticals

Nanoparticles encapsulating enzymes prepared via w/o or w/o/w emulsifications are quite susceptible to denaturation, aggregation, oxidation, cleavage, especially at the aqueous phase solvent interface. This obstacle has been overcome by the addition of stabilizers like carriers proteins (e.g. albumin), surfactants during primary emulsification or by adding molecules like mannitol, trehalose to the protein phase. Three model enzymes i.e. L-asparaginase, catalase and glucose oxidase were encapsulated in poly(3-hydroxybutyrate-co-3-hydroxyvalerate via w/o/w emulsification (Baran et al., 2002) The enzyme activity was increased upon usage of low molecular weight PHBV and adjusting second aqueous phase to isoelectric point of proteins enhanced the encapsulation yields of catalase and L-asparaginase. L-asparaginase was also encapsulated in PLG nanospheres via w/o/w emulsification by (Gasper et al., 1998). They investigated the influence of molecular weight of copolymer and presence of carboxyl-end groups in copolymer chains on the physicochemical and *in vitro* release characteristics of the nanoparticles. Particles having higher molecular weight PLG displayed larger sizes, higher loading and slower release rates than particles made from low molecular weight PLG. Moreover, nanoparticles made of PLG with free carboxyl-end groups showed high protein loading (4.86%) and continuous delivery of active for about 20 days. α-Chymotrypsin (proteolytic enzyme) was encapsulated into PLGA microparticles by w/o/w double emulsion technique (Pérez-Rodriguez et al., 2003). It was observed that interface induced protein aggregation and inactivation could be reduced by co-dissolving it with maltose and poly ethylene glycol in the primary aqueous phase. (Liu et al., 2005c) loaded lysozyme into PLA microparticles by combining Shirasu porous glass membrane (SPG) emulsification and w/o/w double emulsification techniques. They observed that SPG gave higher encapsulation efficiency than stirring method. In another work, lysozyme were loaded into PLGA microparticles via the same w/o/w double emulsion technique to find out the effect of emulsification of lysozyme solutions with methylene chloride on the structural integrity and recovery of active (van de Weert et al., 2000). Another enzyme Staphylokinase variant K35R (DGR) was loaded into PLGA microparticles via double emulsion technique (He et al., 2006). Results showed that encapsulation efficiency was enhanced from 7% to 78% upon coencapsulation of 2% PVA and introduction of 2.5% NaCl into the external aqueous phase of the w/o/w emulsion.

Double emulsion technique has also been applied to encapsulation of antibodies. Poor stability and low efficiency are major hurdles in the formulation of engineered monoclonal antibodies (mAbs) for different therapeutic uses, which may be severer for application to encapsulation into nanoparticles. Son et al.(2009) investigated the formulation and stabilizing conditions for encapsulation of mAb (3D8 scFv) into PLGA nanoparticles via double emulsion. It was concluded that mannitol was the most suitable stabilizer to retain stability and activity of 3D8 scFv. Immunoglobulin G is an antibody contributing to 75% of serum immunoglobulin in humans and it provides protection to fetus in uterus This antibody was encapsulated into PLGA microparticles via s/o/w double emulsion technique (Wang et al., 2004). They investigated the stabilizing effects of different excipients during the period of protein atomization by spray freeze drying and subsequent encapsulation into the particles. They observed that double emulsion solvent evaporation process inactivated approximately 80% of the total IgG. More recently another monoclonal antibody Anti-Annexin A2 (AnxA2) was encapsulated via w/o/w emulsion into microparticles (Gdowski et al., 2015). Nanoparticles were monodispersed, 250 nm in size and had encapsulation efficiency of 18.7%. Particles exhibited sustained release and maintained their functionality upon release. In one study, malarial antigen SPf66 was encapsulated into PLGA microparticles via w/o/w emulsification (Igartua et al., 2008b). They observed the effect of gamma-irradiation on the biopharmaceutical properties of the particles and found out that the irradiation exposure did not affect the integrity of SPf66. Moreover, *in vivo* activity of malarial antigen was retained for week 27. (Wei et al., 2008) used w/o/w emulsification and premix membrane emulsification to encapsulate hepatitis B surface antigen. They observed that usage of diblock copolymer PLA-mPEG provided higher encapsulation efficiency as compared to using triblock copolymer PLA-PEG-PLA. Double emulsion solvent evaporation technique has been applied to encapsulation of other miscellaneous biopharmaceuticals as summarized in Table 5.

Table 5

Various biopharmaceuticals encapsulated via double emulsion technique

Biopharmaceutical	Polymer	Type of emulsion	Observation	Reference
Recombinant human erythro-poietin (rhEPO)	PLGA	W/O/W	Reduction of rhEPO aggregates by using cyclodextrins, arginine, BSA	(Morlock et al., 1997)
Tetanus toxoid	PLA	W/O/W	Max. entrapment efficiency when serum albumin, sucrose, sodium bicarbonate used in internal aqueous phase and sucrose in external aq. phase	(Katare and Panda, 2006)
Luteinizing hormone releasing hormone (LHRH) antagonist	PLGA	double emulsion single solvent evaporation (S/O/O)	E.E influenced by LA/GA ratio of PLGA, salt conc., solvent mixtures and preparation method	(Du et al., 2006)
LHRH antagonist (orntide acetate	PLA and PLGA	W/O/W	Peptide binding to microparticles dependent on pH; higher in phosphate buffer pH 7.4 than in acetate buffer pH 4.0	(Kostanski et al., 2000)
Glial cell-line derived neurotrophic factor (GDNF)	PLGA	W/O/W	Diameter between 8-30µm & E.E 50-100%	(Garbayo et al., 2008)
Exenatide	Polysacchari-de micropart-icle (PAMs)	W/O/W	E.E 60-90% Sustained release for 21 days	(Yang et al., 2009)
Exenatide	PLGA	W/O/W	Significant hypoglycemic activity within 1 month	(Liu et al., 2010)
Glucagon like peptide-1 (GLP-1)	PLGA	W/O/W	Optimized formulation achieved, controlled release *in vivo* for 28 days	(Yin et al., 2008)
Bovine Hb	PELA	W/O/W	E.E 90%, Size 3-5µm	(Qiu et al., 2004)
Basic fibroblast gro-wth factor (bFGF)	PLGA	W/O/W	In vitro release from particles 72-47%in 11 days, bioactivity of bFGF preserved	(Shen et al., 2008)
Bovine lactoferrin	Poloxamer	W/O/W	Developed an optimized nanoemulsion formulation for mouth wash elixir	(Balcão et al., 2013)

5. Theranostic applications

The development of agents for simultaneous diagnosis and treatment of various diseases has gone through extensive investigations in recent years for biomedical applications. These multifunctional theranostic agents allow for feedback mechanism to establish the localization of drug, release of drug, disease phase and efficacy of the treatment (Ahsan et al., 2013; Eissa, 2014; McCarthy, 2010). At present most of the researches in theranostic have been focused primarily on oncology, since, cancer is one of the most fatal diseases and currently the main cause of morbidity and mortality. It is assumed that a by this approach cancer can be managed timely with reduced cost (Ahmed et al., 2012a). Though, double emulsion technique has been frequently used for encapsulation of proteins, peptides hydrophilic and hydrophobic drugs, however limited work has been done in encapsulation of diagnostic agent and therapeutic agent simultaneously via double emulsification process, to the best of our knowledge. Some of the published studies are summarized here.

Theranostic particles fabricated from different polymers have been used for better diagnosis and improved drug delivery, by several groups of researchers such as Yang et al (Yang et al., 2010) fabricated polymer wormlike vesicles loaded with loaded with superparamagnetic iron oxide (SPIO) nanoparticles (as MRI contrast agent) and anticancer drug doxorubicin (DOX) for targeted cancer therapy and MR imaging. The calculated SPIO nanoparticles (NPs) loading content in the vesicles was about 48.0 wt%, while The DOX loading level for these vesicles was about 9.0 wt%. This type of nanocarriers has the advantage that the SPIO and DOX loading amount can be easily changed by adjusting various process parameter during preparation and by changing the chemical structure of the triblock copolymers. This theranostic vesicle nanocarrier system was established to be very efficient, which can provide controlled and targeted drug delivery to the tumor as well as it can be used as an efficient MRI contrast agent, thus providing targeted cancer therapy and diagnosis simultaneously. Similarly, Park et al (Park et al., 2012) incorporated dexamethasone in PLGA nanoparticles and drug-loaded particles were then complexed with (PEI)/siRNA. Co-delivery approach of siRNA and dexamethasone was used in the treatment of rheumatoid arthritis. Almost over 50% of dexamethasone was loaded onto PLGA NPs. And, Ngaboni et al (Ngaboni Okassa et al., 2005) prepared biodegradable sub-micron PLGA particles by double emulsion technique, loaded with magnetite/maghemite nanoparticles (Mag NPs) for intravenous drug targeting. Mag NPs were incorporated in the inner

aqueous phase and the final particle size was found to be 268-327 nm with the magnetite entrapment efficiency up to 60%. Several biomolecules have been also encapsulated via double emulsification for theranostic applications such as Ibraheem et al (Ibraheem et al., 2014b) entrapped human albumin protein into biodegrable polymer (PCL) along with fluorescence active molecule i.e. FITC-BSA (albumin-fluorescein isothiocyanate labeled bovine serum albumin). They incorporated human albumin into inner aqueous phase and emulsified in DCM solution containing polycaprolactone polymer using ultra-turrax hominization. The encapsulation efficiency of albumin was sufficiently high (up to 95%) and albumin-loaded particle was about 340 nm. And the confocal microscopy revelled that all the loaded molecules were evenly distributed in the polycaprolactone matrix.

Another study performed by Ahmed et al (Ahmed et al., 2012b) demonstrates the effective loading of iron oxide and a hydrophilic model drug (stilbene) into polymeric submicron particles for in vivo theranostics. They incorporated the hydrophilic model drug i.e stilbene in the inner aqueous phase (W1) of double emulsion and homogenized with polycaprolactone dissolved in DCM (oil phase). Subsequently the primary emulsion was dispersed in 0.5 % PVA solution (outer aqueous phase). Additionally, organic iron oxide nanoparticles were incorporated in oil phase to be used as MRI contrast agent for imaging. The results showed that iron oxide particles were proper encapsulated by biodegradable polymeric shell of polycaprolactone and the average size of loaded particles were about 300-400 nm. Such hybrid particles owning the dual properties of diagnosis and therapy can be used for co-delivery of multimodal diagnostic agents and a variety of drugs in treatment of fatal disease such as cancer. Some examples of theranostic particle encapsulated via double emulsion process are listed in Table 6.

The double emulsion technique has also been used for particle preparation with dual targeting ability (magnetic and molecular targeting) and co-delivery of hydrophilic and hydrophobic drugs simultaneously. Chiang et al (Chiang et al., 2014b) encapsulated hydrophilic doxorubicin (DOX) and hydrophobic paclitaxel (PTX) in nanoparticles along with superparamagnetic iron oxide (SPIO), and these particles were further conjugated with trastuzumab (monoclonal antibody) in order to specifically target the HER-2 positive cancer cells. DOX was added into the inner hydrophilic phase I (2 wt% PVA solution) while PTX was added to the hydrophobic phase II (chloroform containing 5 mg SPIO) and sonicated to form the primary emulsion (W/O). The primary emulsion homogenized using sonication with

hydrophobic phase III to form a double emulsion (W/O/W). Afterward, organic solvent was removed and particles were collected by centrifugation at 9000 rpm and redispersed in DI-water. The average particles were found to be 174 nm, while the encapsulation efficiency of PTX and DOX in PTX-DOX-nanoparticles were 91% and 72% respectively. They reported that HER-2 positive antigens showed excellent binding ability toward the trastuzumab-conjugated nanoparticles, thus, demonstrating the targeting ability of these nanoparticles. When the magnetic field was applied on the tumor site, there was an increase in amount of particles accumulation. By using magnetic targeting, about 25.8% of PTX and 20% of DOX from the initial dose were accumulated in the tumor for trastuzumab-PTX-DOX-nanoparticles, which was 2.47-fold higher compared to the particle without trastuzumab conjugation. Drug delivery using dual targeting may provide a more specific accumulation of drug-loaded particles at the desired tumor site, which can contribute to lower drug doses and thus reduce the side effects of cancer therapy. Shen et al (Shen et al., 2013) prepared PLGA nanoparticles by using double emulsion technique. Two hydrophilic drugs, doxorubicin and verapamil (VER) were initially combined with chitosan shell coated on magnetic nanoparticles (MNPs), which were then incorporated into PLGA nanoparticles to be used for cancer therapy through dual-drug delivery system (DDDS). Additionally, a tumor-targeting ligand was also conjugated onto the end carboxyl groups on the PLGA-NPs. From morphological observations, drug loaded NPs were found to be spherical and with a narrow range of distribution of the particle sizes (about 130-140 nm). While, the entrapment efficiencies of DOX and VER were about 74.8 and 53.2 wt % respectively. In vitro drug release behaviors were evaluated under dialysis condition at 37 °C in a simulative normal body fluid (50 mM PBS, pH 7.4) and an acidic environment (50 mM PBS, pH 5.3). The cumulative release of DOX and VER at 37 °C was 29.0 and 41.3% respectively at pH 5.3; and 25.0 and 34.5% respectively at pH 7.4. It was demonstrated that the intelligent DDDS could significantly inhibit both, the growth of tumor as well as DOX-induced cardiotoxicity in mice, and potentially offer an approach for safe cancer therapy.

Table 6
Theranostics particles applications and composition of various components used in their preparation.

Actives/probes (in the inner aqueous phase)	Solvent	Polymer	Stabilizer	Particle Size	Objectives	References
MagNP	Methylene chloride	PLGA	PVA 0.3% (w/v)	268-327 nm	Encapsulation of magnetic particles for intravenous drug targeting.	(Ngaboni Okassa et al., 2005)
Iron oxide, stilbene	DCM	PCL	PVA 0.5%	300-400 nm	Preparation of hybrid particles for in vivo theranostics	(Ahmed et al., 2012b)
HSA, FITC-BSA	DCM	PCL	PVA	340 nm	Albumin encapsulation for in vivo imaging applications	(Ibraheem et al., 2014b)
Dexamethasone	Methylene chloride	PLGA	PVA 1% (w/v)	90 nm	Treatment of rheumatoid arthritis	(Park et al., 2012)
SPIO	Chloroform	FA-PEG_{114}-PLA_x-PEG_{46}-acrylate	PVA 0.1%	100-200 nm	Cancer diagnosis and therapy	(Yang et al., 2010)
DOX	PTX and SPIO in chloroform	PMAA	PVA 2%	174 nm	Dual targeting and co-delivery of hydrophilic and hydrophobic drugs	(Chiang et al., 2014b)
Carboplatin and GMO-GMPs	Paclitaxel and PLGA in DCM	PLGA	PVA 5%	100 nm	co-delivery of hydrophilic and hydrophobic anticancer drugs and MRI imaging	(Singh et al., 2011)
DOX	Magnetite in chloroform	chitosan	Tween 80, 4%	100-150 nm	Doxorubicin-loaded MNPs for drug delivery	(Balan et al., 2015)
QDs and cetux-imab antibody (targeting agent)	Camptothecin and QDs in acetone	PLGA	PEG 0.1%	178 nm	Multifunctional nanoparticles with in vitro targeted imaging ability and delivery of camptothecin.	(Deepagan et al., 2012)
DOX, VER and MNPs-CA	DCM	PLGA	PVA 5%	130-140 nm	Polymeric MNPs for targeted dual-drug delivery for cancer therapy	(Shen et al., 2013)
Adriamycin or siRNA	Chloroform	mPEG-PLGA-b-PLL	pluronic F-68	169-151 nm	copolymer carrier for adriamycin and siRNA delivery	(Liu et al., 2012)
siRNA	Chloroform	mPEG-PLA	PVA 1%	170-200 nm,	siRNA encapsulation and delivery for cancer therapy	(Yang et al., 2011)

FNPs	Dichloromethane (DCM)	PCL	PVA 0.5%	342-375 nm	FNPs encapsulation as a model contrast agent (Iqbal et al., 2015)

MagNP = Magnetite/maghemite nanoparticles, DOX= Doxorubicin, SPIO = Superparamagnetic iron oxide, FA-PEG_{114}-PLA_x-PEG_{46}-acrylate= Folate-poly(ethylene glycol)-poly(d,l-lactide)-poly(ethylene glycol)-acrylate, HSA= Human serum albumin FITC-BSA= Albumin-Fluorescein isothiocyanate conjugate bovine serum albumin, PTX= Paclitaxel, PMAA= Poly(methacrylic acid), GMO-GMPs= Glyceryl monooleate magnetic nanoparticles, QDs=Quantum dots (tracking agent), PEG= Polyethylene glycol, MNPs-CA= citric acid modified MNPs, VER= Verapamil, mPEG-PLGA-b-PLL= monomethoxy (polyethylene glycol)-poly (d,l-lactide-co-glycolide)-poly (l-lysine), mPEG-PLA= Poly(ethylene glycol)-*b*-poly(*d,l*-lactide), FNPs= Fluorescent nanoparticles.

6. Conclusion

Double emulsions have many potential applications. In biomedical field, it can be used in drug delivery systems for encapsulation of both hydrophobic as well as hydrophilic active medicaments, cosmetics, foods, imaging agents and other high value products. In modern drug delivery system, it is a challenge to administered hydrophilic biomolecules such as nucleic acids and proteins. Because of their water solubility, they tend to diffuse into continuous aqueous phase during emulsification process. However, the double emulsion technique has been extensively used for the encapsulation highly water soluble compounds including protein and peptides. Encapsulation of these compounds prevents its degradation, control the rate and extent of release and enhances the loading efficiency. Nanoparticles made by double emulsion solvent evaporation method are excellent carriers for delivery of hydrophilic molecules such as proteins, peptides and variety of pharmaceutical and biopharmaceutical compounds. It offers stability and controlled release of encapsulated molecules. Beside several advantages, double emulsion process has a drawback of shear force used for homogenization of inner aqueous phase containing biomolecules in the organic phase. Which can damage the integrity of biomolecules, thus leads to loss of its biological activity. Moreover, the organic solvent can adversely affect the structure of biomolecules during homogenization. This damage can be minimized by condensation of biomolecules with cationic polymers, in order to reduce its size and preserve its biological activity. Consequently, the research works reported in this review give information about different polymers, stabilizers and solvents that can be used in this technique, the effects of process parameters and the application of this technique in encapsulation of various pharmaceutical and biopharmaceuticals.

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PART III

EXPERIMENTAL STUDY

III.1. Systematic study of double emulsion prepared using ultra-turrax

General summary

Different Polymer-based encapsulation techniques have been widely studied by different research groups, including: nanoprecipitation method, emulsion diffusion method, double emulsion evaporation method, emulsion coacervation method and layer by layer assembly method. In this work, we studied the effects of various parameters on the particle's properties including, particle size, zeta potential and morphology, prepared by emulsion solvent evaporation techniques (DESE). Particles were prepared by DESE process in two steps: in first step, inner aqueous phase (W1) was homogenized with organic phase (O) containing polymer, to form first emulsion. This was followed by second step, in which, first emulsion was homogenized with outer aqueous phase using high shear ultra-turrax homogenizer for specific time and speed to achieve double emulsion (W1/O/W2). Subsequent evaporation of organic solvent from dispersed phase has led to particulate suspension at the end. Polycaprolactone (polymer) dissolved in dichloromethane (solvent) was used as organic phase (O), while polyvinyl alcohol solution was used as outer aqueous phase (W2).

As a general tendency, zeta potential of all prepared particles was found to be constant at different pH (3, 5, 7, 9 and 11) for all samples, which means that changing of PCL particles preparation conditions have no significant effect on the zeta potential. No change in zeta potential can be attributed to non-charged character of polycaprolactone particle as already reported in literature. The effects of different parameters on particle size was studied and found that: stirring speed of homogenizer has significant effect on the particle size in the second step of emulsification process and almost small size particles were obtained at high stirring speed; while in the first step, there was insignificant effect of stirring speed.

Similarly, the effect of stirring duration (time) on particle's size was investigated, and results showed that with an increase in stirring time there is significant decrease in particle size during the second step; however its effect was insignificant during the first step of emulsification. It was found that the increase in polymer amount leads to large size particles, and with increase in outer aqueous phase volume a slight decrease in particles size was reported. The decrease in PVA concentration below 0.2% leads to large particle formation, while above 0.2% PVA concentration it showed no significant effect on particle's size. From SEM images observation, it was found that the surface of obtained microparticles can be assumed to be spherical with smooth surface, having narrow size distribution. Compare to the results from

Laser Diffraction Particle Size Analyses (LS-13 320), the particles measured with SEM were slightly smaller in size this may be due to contraction induced by drying during evaporation of solvent.

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Effects of process parameters on the colloidal properties of polycaprolactone microparticles prepared by double emulsion like process

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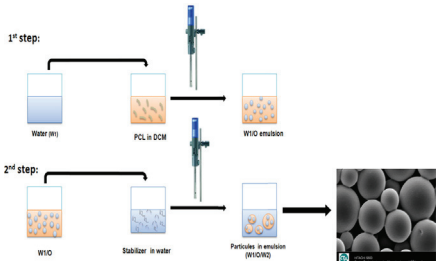
HIGHLIGHTS

- Formulation of polymeric particles through double emulsion like process.
- Effects of process parameters on particles colloidal properties.
- Both agitation time and speed affect the final average particles size.
- The use of high amount of polycaprolactone leads to large particles size.

GRAPHICAL ABSTRACT

In the first step, inner aqueous phase (W_1) was added to dichloromethane (DCM) containing polycaprolactone (PCL) polymer and homogenized to form primary emulsion (W_1/O). In the second step, the primary emulsion was emulsified in the outer aqueous phase (W_2) containing polyvinyl alcohol (PVA) as stabilizer using ultra-turrax to have double emulsion ($W_1/O/W_2$). SEM micrograph shows the particles morphology.



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ABSTRACT

Preparation of polycaprolactone (PCL) based microparticles by double emulsion solvent diffusion like process was studied in this work. The double emulsion was prepared in two steps. In first step, the inner aqueous phase (W_1) was added to dichloromethane (DCM) solution containing PCL and homogenized to form primary emulsion (W_1/O). In the second step, the primary emulsion (W_1/O) was emulsified with the outer aqueous phase (W_2) containing polyvinyl alcohol (PVA) as stabilizer using ultra-turrax at a specific speed and time in order to achieve the double emulsion ($W_1/O/W_2$). Effects of various parameters such as stirring time and speed, polymer amount and the volume fraction of each phase on hydrodynamic particle size, size distribution and zeta potential were investigated.

As a general tendency, zeta potential of all prepared particles was found to be constant irrespective of investigated parameter. Whereas, the increase in polymer amount leads to large particles size and lowest sizes were obtained when high stirring speed was used during the second emulsification step.

1. Introduction

The key point for pharmaceutical researches is to fabricate pharmaceutical drug delivery system that enhance drug efficiency and diminish the undesirable effects [1,2]. Encapsulation technique is one of the techniques, which are used effectively for achieving this aim. It can be identified as the technique by which the active material is walled or coated by a supported material, that shielding it from the external environment [3–5]. It has found many

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applications in different fields like pharmaceuticals [6,7], cosmetics [8,9], foods [10], diagnoses [11] and printing industries [12]. For instance, in pharmaceutical field, drug encapsulation can protect the active ingredient against harsh biological environment; mask the unpleasant taste and smell of the drug. Additionally, it can be used in drug delivery system for targeting the drug to specific site and to control the drug release [13,14].

Encapsulation technique, based on the method of preparation, may lead to the formation of spheres or capsules [15]. Generally, spheres are more stable than capsules. As a result, the drug liberation from the spheres is slower; therefore, spheres can be employed when a prolonged drug release is needed [16]. Encapsulation can be achieved by using various strategies [17]. Among these strategies, biopolymer-based encapsulation techniques are the most appropriate in pharmaceutical domain [18–20], due to the biodegradability and biocompatibility of polymers which is suitable for in vivo applications [21]. Polymer-based encapsulation techniques have been well studied and reviewed by various researcher teams [22–24], including: nanoprecipitation method, emulsion diffusion method, double emulsification method, emulsion–coacervation method, polymer-coating method, and layer-by-layer (LBL) assembly method.

Here we studied particles preparation by double emulsification method (multiple emulsion method); as this technique can be used to encapsulate both hydrophilic and lipophilic substances [25]. In addition, it is suitable for water-soluble fragile drug materials which are very sensitive to the high temperatures, such as nucleic acids and protein [26–28].

Multiple emulsions were described for the first time in 1925 by Seifriz [29], it can be identified as complex [30] polydispersed systems, in which the dispersed phase is itself an emulsion, that has inner phase of the same nature of the double emulsion [32]. In double emulsion two types of emulsion (water in oil and oil in water) are found simultaneously [33] using two types of surfactants (lipophilic and hydrophilic) for stabilizing them. Garti has identified these systems as "emulsions of emulsions" [34]. The presence of two types of emulsion simultaneously in one system, gives it the properties of the two emulsions. The multiple emulsions may be classified into two types: water in oil in water (W/O/W) and oil in water in oil (O/W/O) [35], the first type (W/O/W) is frequently employed for pharmaceutical purposes [36]. The composition of double emulsions and their properties make them promising systems that have potential applications in various fields for example, in pharmaceutical [37], in food industry and in cosmetics [38]. In spite of all these important properties, the applications of multiple emulsions are still limited because of their inherent thermodynamic instability. In recent years, great efforts have been made to improve the multiple emulsions properties, especially, increasing the emulsion stability, decreasing and homogenizing the emulsion droplets size. Many factors can affect the double emulsion stability such as, method of preparation, type of oil phase, type and concentration of emulsifiers and so on [39].

The aim of the present work is to study for the first time the influence of various parameters on colloidal properties (i.e. particles size, zeta potential, size distribution and morphology) of the final particles prepared by double emulsion solvent diffusion like process like. The parameters investigated were; polymer content, concentration of stabilizer (PVA), inner and outer aqueous phase volumes, time and speed of stirring in 1st and 2nd steps of emulsification process.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL) (Mw = 14,000 g/mol), polyvinyl alcohol (PVA) (Mowiol® 4-88, Mw = 31,000 g/mol), and dichloromethane (DCM) were obtained from Sigma–Aldrich, Germany, distilled water. Ultra-turrax (T-25 basic IKA-WERK), Laser Diffraction Particle Size Analyzer LS 13 320 (Beckman Coulter, USA). Field Emission Scanning Electron Microscope (S-800 Hitachi, Japan). Zetasizer (Nano-ZS, Malvern, UK). Analytical balance (Acculab ALC-110.4) was supplied by Sartorius Group, Germany.

2.2. Preparation of PVA solution

To be used as outer aqueous phase, 0.5% PVA solution was prepared by adding 2.5 g of PVA in 500 ml flask and distilled water was added to make up the volume, and then PVA was dissolved using magnetic stirrer under heating at 60 °C for 40 min to obtain a clear PVA solution.

2.3. Preparation of particle by double emulsion like process

The microparticles were fabricated by double emulsion solvent diffusion method. Two-step emulsification process was used; the primary emulsion (W_1/O) was dispersed as small droplets in the outer aqueous phase (W_2) with the help of ultra-turrax stirrer. PVA was used as emulsion stabilizer in the outer aqueous phase.

2.3.1. Preparation of primary emulsion (1st step)

In the first step, in order to make the primary emulsion (W_1/O); 3 g of polycaprolactone (polymer) was dissolved in 12 ml of DCM and shacked on rolling shaker until form a clear solution. And then 1.5 ml of distilled water was added in PCL solution, this mixture was homogenized properly using ultra-turrax (T-25 basic IKA-WERK) at a specific speed and for a specific time (Table 2) to have the first emulsion (W_1/O).

2.3.2. Preparation of double emulsion (2nd step)

In the second step, the primary emulsion (W_1/O) was added in the outer aqueous phase (W_2) containing 0.5% PVA as stabilizer. This mixture was homogenized by using ultra-turrax (T-25 basic IKA-WERK) at specific speed for specific time (Table 2), to achieve the double emulsion ($W_1/O/W_2$). Finally, the diffusion of organic solvent from dispersed primary emulsion droplets to outer aqueous phase (W_2), resulted into the formation of solidified suspended polycaprolactone (PCL) particles. In 2nd step we used excess of outer aqueous phase (W_2) in order to facilitate the diffusion of organic solvent from PCL particle to outer aqueous phase. Fig. 1 represents the two steps process for microparticles preparation by double emulsion solvent diffusion like process.

2.4. Reference emulsion composition

The value of parameters indicated in Table 1 was used to prepared reference emulsion. A set of experiments were performed,

Table 1
Composition of reference parameters of double emulsion.

Primary emulsion's parameters (1st step)				Double emulsion's parameters (2nd step)			
Amount of PCL (g)	Volume of inner phase w_1 (ml)	Stirrer speed (rpm)	Stirrer time (min)	PVA (%, w/v)	Stirrer speed (rpm)	Stirrer time (min)	Volume of outer phase w_2 (ml)
3	1.5	17,500	5	0.5%	21,500	5	150

Table 2
Parameters (changed and fixed) in different set of experiments. The "bold" values show the parameters, which were changed in their respective recipes.

Studied parameters	Primary emulsion's parameters (1st step)				Double emulsion's parameters (2nd step)				Average particle size (μm)
	PCL (g)	Inner phase W_1 (ml)	Stirring time (min)	Stirring speed (rpm)	PVA (%)	Outer phase W_2 (ml)	Stirring time (min)	Stirring speed (rpm)	
Stirring speed for 1st emulsion	3	1.5	5	**6500**	0.5	150	5	21,500	6.5
	3	1.5	5	**9500**	0.5	150	5	21,500	6.5
	3	1.5	5	**13,500**	0.5	150	5	21,500	6.8
	3	1.5	5	**17,500**	0.5	150	5	21,500	6.7
Stirring speed for 2nd emulsion	3	1.5	5	17,500	0.5	150	5	**6500**	37.9
	3	1.5	5	17,500	0.5	150	5	**9500**	21.8
	3	1.5	5	17,500	0.5	150	5	**13,500**	13.7
	3	1.5	5	17,500	0.5	150	5	**21,500**	7
Stirring time for 1st emulsion	3	1.5	**2**	17,500	0.5	150	5	21,500	7.7
	3	1.5	**4**	17,500	0.5	150	5	21,500	7
	3	1.5	**6**	17,500	0.5	150	5	21,500	7.8
	3	1.5	**8**	17,500	0.5	150	5	21,500	7.3
Stirring time used for 2nd emulsion	3	1.5	5	17,500	0.5	150	**2**	21,500	9.9
	3	1.5	5	17,500	0.5	150	**4**	21,500	8.3
	3	1.5	5	17,500	0.5	150	**6**	21,500	7.7
	3	1.5	5	17,500	0.5	150	**8**	21,500	6.6
Concentration of stabilizer (PVA)	3	1.5	5	17,500	**0.05**	150	5	21,500	12.7
	3	1.5	5	17,500	**0.1**	150	5	21,500	8.65
	3	1.5	5	17,500	**0.2**	150	5	21,500	8.5
	3	1.5	5	17,500	**0.5**	150	5	21,500	10.6
	3	1.5	5	17,500	**1**	150	5	21,500	9.1
	3	1.5	5	17,500	**2**	150	5	21,500	10.6
Amount of polymer used (PCL)	**1**	1.5	5	17,500	0.5	150	5	21,500	4.2
	2	1.5	5	17,500	0.5	150	5	21,500	6
	3	1.5	5	17,500	0.5	150	5	21,500	8.5
	4	1.5	5	17,500	0.5	150	5	21,500	11.6
Volume of inner aqueous phase (W_1)	3	**1**	5	17,500	0.5	150	5	21,500	8.7
	3	**1.2**	5	17,500	0.5	150	5	21,500	9.6
	3	**1.5**	5	17,500	0.5	150	5	21,500	9.2
	3	**2**	5	17,500	0.5	150	5	21,500	8.6
Volume of outer aqueous phase (W_2)	3	1.5	5	17,500	0.5	**50**	5	21,500	10.8
	3	1.5	5	17,500	0.5	**100**	5	21,500	7.9
	3	1.5	5	17,500	0.5	**150**	5	21,500	9.8
	3	1.5	5	17,500	0.5	**200**	5	21,500	8.9

to study the effect of different parameters on the characteristics of the particles prepared via double emulsification by changing only one parameter at a time and keeping all other parameters fixed. For example, to study the parameter "polymer amount" four samples were prepared with different amount of PCL i.e. 1 g, 2 g, 3 g and 4 g while keeping all other conditions (stirring time, stirring speed, phase volume, etc.) constant (Table 1); and particles size were measured for all samples.

2.5. Particles size measurement

The particles size and size distribution were studied using Beckman Coulter LS 13 320 Laser Diffraction Particles Size Analyses. The samples were added drop by drop into the sample cell comprising continuous phase (deionized water), the pump speed was adjusted to 20% for appropriate mixing of sample. When the obscuration reached 8–9% then sample analysis was started.

2.6. Zeta potential

Zeta potential was measured at different pH values at 25 °C, for this purpose solution of 1 mM (milli Molar) concentration of NaCl was prepared and its pH was adjusted to different values like 3, 5, 7, 9 and 11. After that each sample's zeta potential was measure at these pH values with help of Zetasizer (Nano-ZS, Malvern).

2.7. SEM observation

Scanning Electron Microscopy, SEM, was performed with a Hitachi S800 FEG microscope at the "Centre Technologique des Microstructures" (CTμ) at the University of Lyon (Villeurbanne, France). A drop of diluted aqueous suspension of microparticles was deposited on a flat steel holder and dried at room temperature. The sample was finally coated under vacuum by cathodic sputtering with platinum. The samples were observed by SEM under an accelerating voltage of 15 kV.

3. Results and discussion

The purpose of this work was to study the effects of different parameters such as polymer amount, stabilizer concentration, stirring time and stirring speed used, on the size of particles prepared by double emulsion like process. Double emulsion $W_1/O/W_2$ was prepared by a two-step emulsification process using PVA as stabilizer in the second step.

3.1. Effects of different parameters on particle size and size distribution

3.1.1. Effect of stirring speed

Since the emulsion is a mixture of two or more immiscible liquids so, in order to make it uniformly dispersed, it is necessary

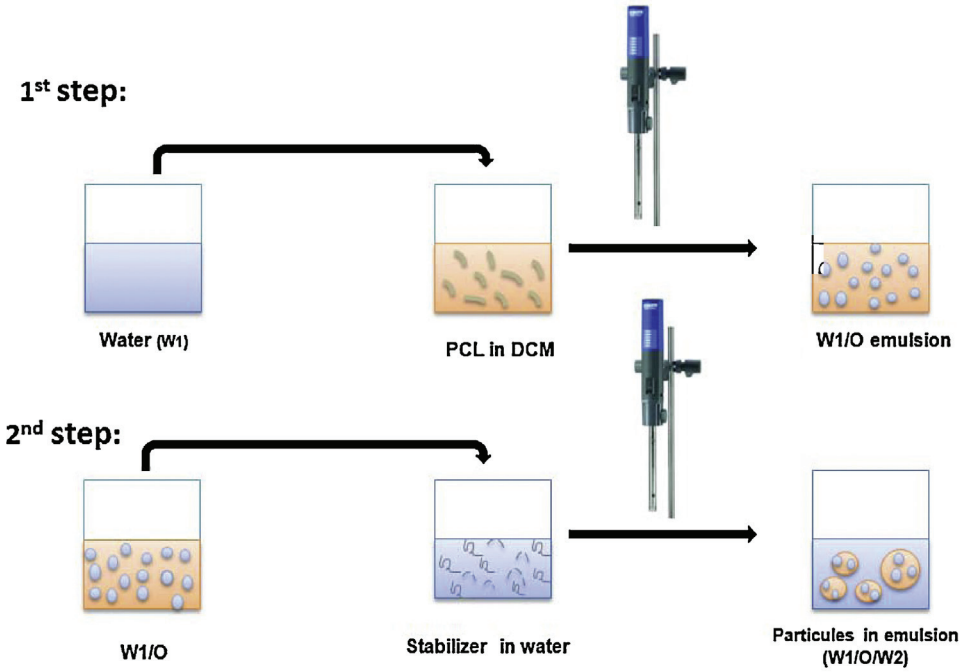

Fig. 1. Schematic illustration of a two-step process in formation microparticles via double emulsion solvent diffusion like process.

to provide this system with the adequate energy [40], either by high stirring or by ultrasound system [34]. However, the agitation used to provide the needed energy should not be very severe, especially during the second step of emulsion preparation, because it may rupture the droplets obtained from the first step [34,36]. Yang et al. have found that the stirring speed is a governing factor in size determining of the particles prepared via double emulsification technique [40]. The work of Yang's team demonstrates that, high stirring speed produces very small size particles because of the dismantling of second emulsion into smaller droplets. However, the final particles yield is low, due to the breaking down of the resultant microspheres [40]. In this work the necessary energy was supplied to the system via high stirring using ultra-turrax and the influence of stirring speed on the final particles size was studied in both steps of emulsification. In first step, the stirring speed used to prepare the first emulsion was changed only, while stirring speed of second emulsion and other conditions were kept constant (Table 2). In the second step, contrary to first step was done, it means that stirring speed of first emulsion with all other parameters were kept constant and only the stirring speed for preparation of second emulsion was changed. The particles size obtained were measured by using *Laser Diffraction Particle Size Analyzer*, the final particles size was in micro range as shown in Fig. 2.

It is obvious from Fig. 2 that the change in stirring speed from 6500 rpm to 9500 rpm during first emulsion preparation has no influence on the size of final particles. And the particles size changed slightly when the stirring speed was further increased i.e. from 9500 rpm to 13,500 rpm, at this stirring speed the resultant particles size was 6.8 μm. By increasing starring speed further to 17,500 rpm, the particle size decreased to 6.7 μm. Fig. 2 shows that, the stirring speed used to prepare the first emulsion has no significant effect on the size of the resulting particles.

In second step of double emulsion, by increasing the stirring speed of ultra-turrax and keeping all other parameters constant, has led to a proportional reduction in the obtained particles size as shown in Fig. 2. Here, the size of particles was 37.6 μm at stirring speed of 6500, and particle size gradually decreased to 21.8 μm, 13.7 μm and 7.0 μm at stirring speed of 9500 rpm, 13,500 rpm and 21,500 rpm respectively. The findings of this study proved that the stirring speed in the second step of double emulsions preparation is a significant factor in particle size determination, i.e. by increasing the stirring speed in 2nd step of emulsion preparation, the size

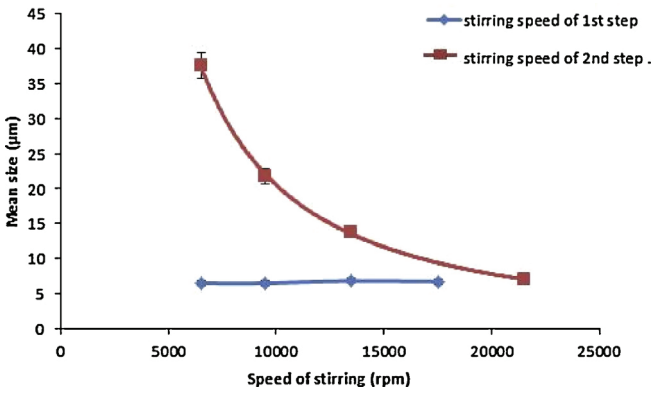

Fig. 2. Effect of stirring speed in 1st step and 2nd step of emulsification on the mean particle size prepared by double emulsion.

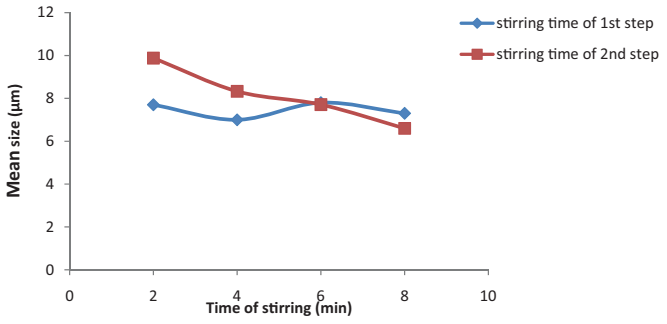

Fig. 3. Effect of stirring time of 1st step and 2nd step of emulsification on the mean particle size.

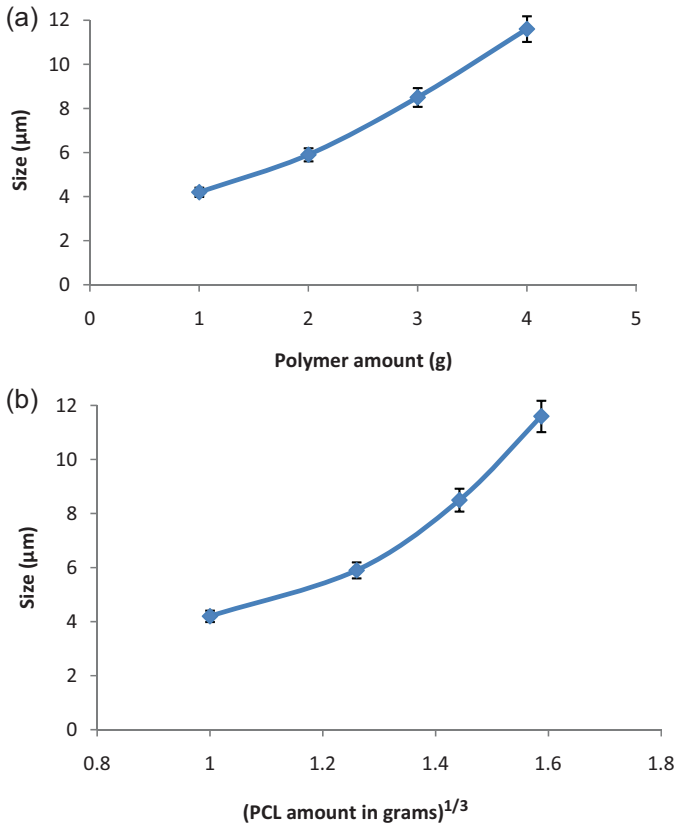

Fig. 4. (a) Effect of polymer amount on the mean particle size. (b). Average hydrodynamic size versus (polymer amount)$^{1/3}$.

of obtained particles decreased proportionally, Which is in accordance with the results reported by Yang [40].

3.1.2. Effect of stirring time

The time duration of providing energy is quite important because short time of stirring grants insufficient energy to the system, while long time of stirring may lead to break down of the emulsion, which in turn will lead to low entrapment efficiency. In this experiment, the stirring time of the first emulsion was changed only, while keeping all other parameters of the recipe constant (Table 2). Four recipes were prepared with different stirring time (2 min, 4 min, 6 min and 8 min) and size of particles achieved were 7.7 μm, 7 μm, 7.8 μm, 7.3 μm respectively (Fig. 3). These results demonstrated that the time duration of stirring for the first emulsion (W_1/O) has no significant effect on the particle size prepared

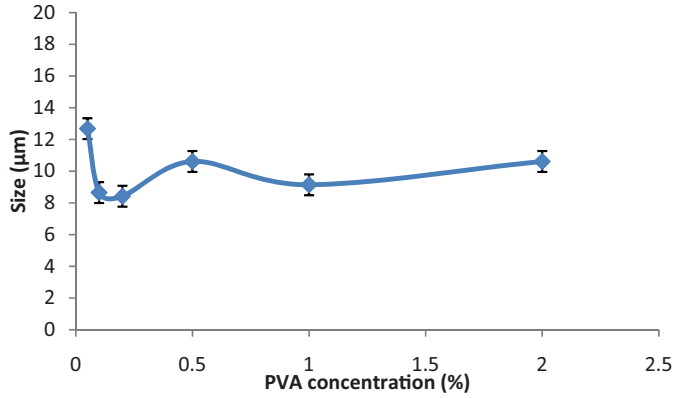

Fig. 5. Effect of stabilizer (PVA) versus mean particle size.

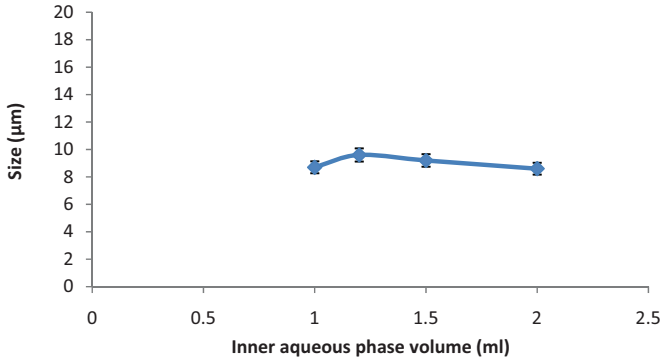

Fig. 6. Inner aqueous phase (W_1) versus PCL mean particles size.

via multiple emulsions solvent diffusion like process as shown in Fig. 3.

The stirring time of the second emulsion was investigated too, by changing the stirring time for the second emulsion and keeping all other conditions unchanged. It was found that the stirring time is an important and influential factor in determining the final particles size in double emulsion. Adequate agitation time is required to obtain small, mono-dispersed particles and to avoid aggregation of the formed particles. However, the stirring time must not exceed a certain limit in order to avoid emulsion spoilage. Here, different stirring time i.e. 2 min, 4 min, 6 min and 8 min were used in 2nd step emulsification, which resulted into particulate dispersion with mean size 9.88 μm, 8.33 μm, 7.7 μm and 6.6 μm, respectively. Fig. 3 shows that there is a gradual decrease in particles size as stirring time increase, this reduction in particle size may be due to supply of input power for longer period of time.

3.1.3. Effect of polymer amount

The polymer amount may be an important factor influencing the particle's characteristics like encapsulation efficacy and particle size. In this work, polycaprolactone was used as polymer and its effects were investigated at different amount of polymer (1, 2, 3 and 4 g) by keeping all other conditions constant. The results obtained (Fig. 4a) implies that at small amount (1 g) of polymer small particles were obtained (4.2 μm) but as the amount of polymer was increased further to 2 g, 3 g and 4 g, the particle size increased significantly which were 5.9 μm, 8.5 μm and 11.6 μm, respectively. These results revealed that by increasing the amount of polymer, the particle size also increases and similar results were reported by Lamprecht et al. [41].

In order to point out the real effect of polymer, we deduced the mathematic relationship between the average particle size (R) and the polymer amount (M), which is; $R = (3/4\pi dN_p)^{1/3} \times (M)^{1/3}$ where (d) is the density of polymer, N_p is the number of particles.

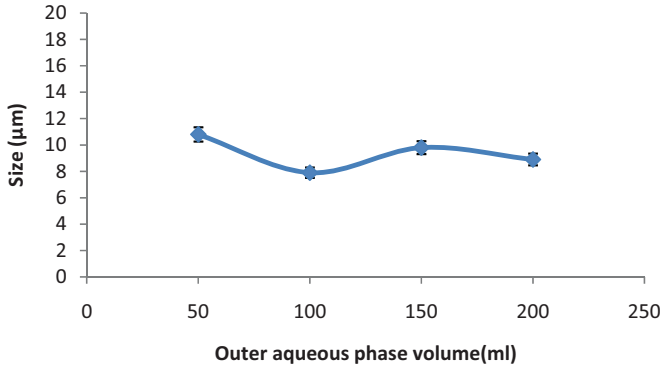

Fig. 7. Effect of outer aqueous phase volume (W_1).

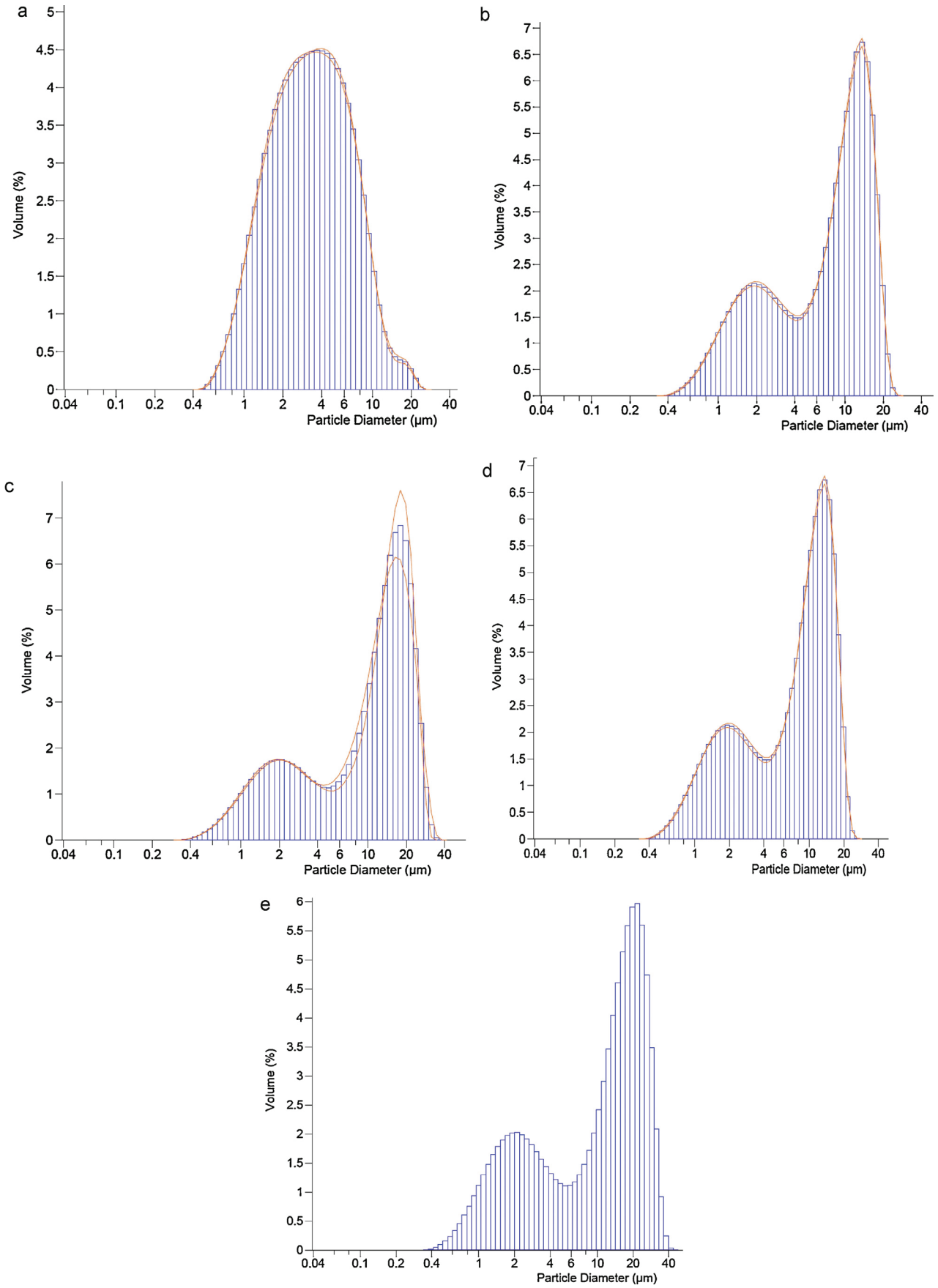

Fig. 8. (a) Particle size distribution of sample prepared using 1 g of PCL. (b) Particle size distribution of sample prepared using 3 g of PCL. (c) Particle size distribution of sample prepared using 4 g of PCL. (d) Particle size distribution of sample prepared using 0.5% g PVA. (e) Particle size distribution of sample prepared using 0.05% g PVA.

Table 3
Zeta potential values (in mV) of different recipes at different pH.

Parameters		Zeta potential (mV) as a function of pH				
Name		pH = 3	pH = 5	pH = 7	pH = 8	pH = 10
Stirring time in 1st step of emulsion	2 min	−0.70	−2.42	−2.41	−3.21	−1.58
	4 min	−1.04	−2.41	−2.71	−3.38	−2.01
	6 min	−1.16	−2.33	−2.64	−2.83	−1.70
	8 min	−0.993	−2.14	−1.69	−3.01	−1.44
Stirring time in 2nd step of emulsion	2 min	1.72	−2.11	−0.65	−2.91	−1.81
	4 min	−1.22	−2.46	−2.32	−3.28	−2.02
	6 min	1.96	−1.38	−0.63	−3.22	−1.33
	8 min	−1.18	−1.87	−2.13	−3.22	−1.62
PCL content	1 g	−1.88	−2.55	−1.65	−3.55	−1.72
	2 g	−0.89	−1.82	−2.44	−2.72	−2.10
	3 g	−1.12	−2.51	−2.37	−3.12	−2.01
	4 g	−0.62	−3.92	−1.83	−3.25	−1.76
PVA (wt./v)	0.2%	0.37	−3.59	−2.85	−3.45	−2.03
	0.5%	−0.98	−2.12	−1.66	−2.23	−2.15
	1%	−0.88	−3.40	−2.33	−3.26	−1.92
	2%	−1.05	−2.53	−1.42	−2.83	−1.70
Stirring speed in 1st step of emulsion	6500 rpm	−0.90	−1.85	−1.52	−1.83	−1.49
	9500 rpm	−0.89	−2.12	−1.28	−2.40	−0.94
	13,500 rpm	−1.14	−1.32	−1.59	−2.48	−1.46
	17,500 rpm	−0.59	−1.79	−1.19	−2.39	−1.86
Stirring speed in 2nd step of emulsion	6500 rpm	−1.56	−1.20	−3.14	−2.11	−1.93
	9500 rpm	−0.82	−1.51	−1.65	−1.35	−0.92
	13,500 rpm	−0.91	−1.73	−2.25	−2.00	−1.44
	21,500 rpm	−0.721	−2.79	−1.87	−3.19	−1.30
Inner (W_1) aqueous phase volume	1 ml	−0.96	−3.38	−1.23	−3.95	−1.77
	1.2 ml	−1.66	−2.75	−2.66	−2.73	−2.25
	1.5 ml	−0.97	−2.52	−2.29	−3.59	−2.00
	2 ml	−1.11	−3.21	−1.59	−3.48	−1.73
Outer (W_2) aqueous phase volume	50 ml	−1.24	−4.67	−3.74	−4.78	−3.32
	100 ml	−1.67	−4.12	−3.16	−3.90	−2.43
	150 ml	−1.02	−3.52	−1.56	−3.57	−2.09
	200 ml	1.00	−3.88	−2.98	−3.97	−2.30

In this relationship, if the number of particles is constant independent of the used polymer amount, the slope that represents the relationship between the particle size and $(M)^{1/3}$ will be straight. But in our case it seems that the slope increases with the increasing of polymer amount in the formulation, as illustrated in Fig. 4b. Such increase in the slope exhibits that using more polymer amount leads to increase the number of obtained particles in the formation. Such increase in the number and in the size of particles can be attributed to the aggregation of unstable particles that can be enhanced by increasing the solid content (polymer amount) and also by low stabilizing efficiency of PVA.

3.1.4. *Effects of stabilizer concentration*

The addition of suitable stabilizer plays a key role in liquid–liquid dispersion [42]. The concentration and type of stabilizer affect the stability and formulation of emulsion. The stability of emulsion is very important because during the evaporation of solvent, the volume of emulsion can be decreases, which in turn increase its viscosity. This may affect the final size of the droplet and may results in the coalescence and aggregation of the droplets during solvent evaporation [43].

The stabilizer stays at oil/water interface during solvent evaporation. In recent times polyvinyl alcohol (PVA) is frequently used as an emulsion stabilizer [44] and its concentration in the external water phase is considered to be vital factor to influence the size of microparticles [40]. Since PVA is a high molecular weight polymer, the presence of PVA in the outermost water phase (W_2) may increase the viscosity of the dispersion phase, resulting in an increased difficulty to reduce the emulsion particles to smaller size [40,45].

In this study, six samples were prepared with different concentration of PVA in the outer aqueous phase (W_2), it was found that by increasing PVA concentration (0.05%, 0.1%, and 0.2%) has led to marked decrease in particle size and minimum size was achieved at 0.2% PVA which was 8.4 μm (Fig. 5). By increasing further the PVA concentration to 0.5%, 1% and 2% the particle size were slightly increased with some variations, which was 10.6 μm, 9.1 μm and

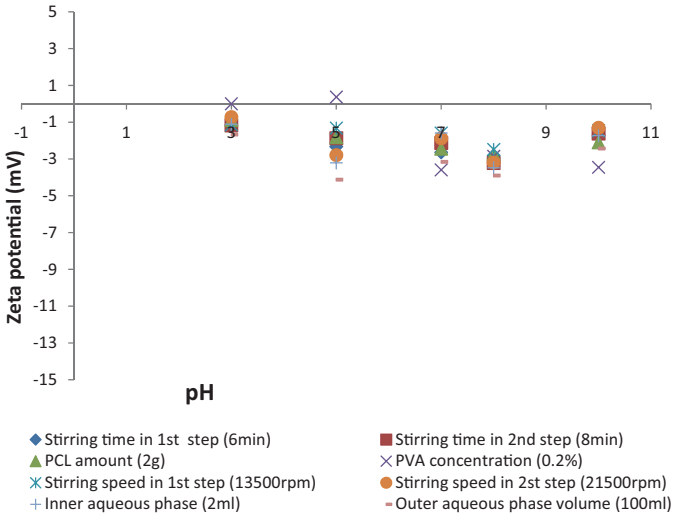

Fig. 9. Zeta potential of PCL microparticles as a function of pH, at different conditions.

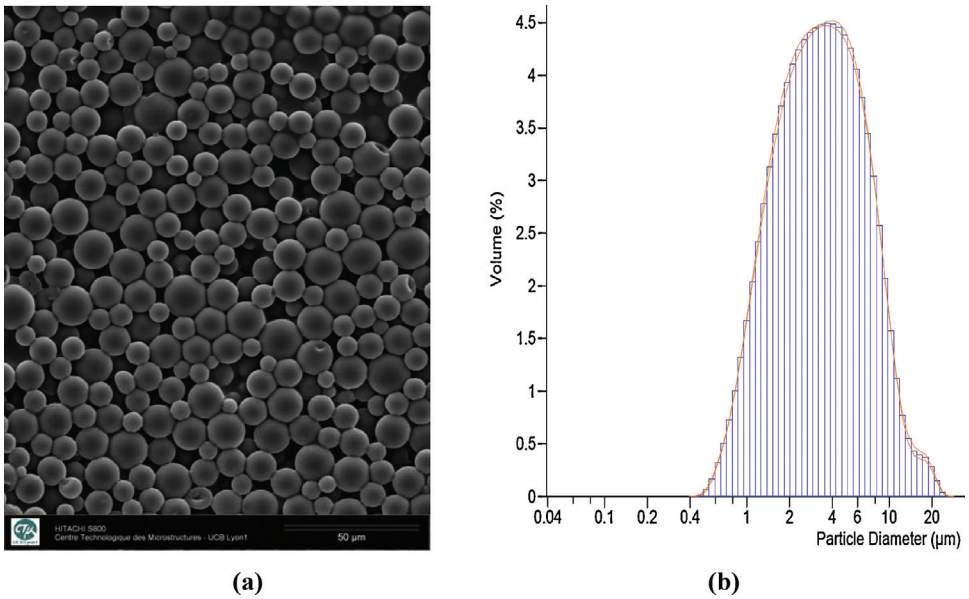

Fig. 10. (a) SEM image of PCL particles prepared by double emulsion like method. The scale bar represents 50 μm. (b) Particle size distribution diameter from Laser Diffraction analysis.

10.6 μm, respectively. Additionally one sample was prepared without PVA in outer aqueous phase (W_2) but in this case no particles were formed and the emulsion was absolutely unstable. The results obtained (Fig. 5) showed that 0.2% PVA was more appropriate in achieving smaller size microparticles in this method.

3.1.5. Effect of inner aqueous phase volume

In this work, we used different volumes of internal aqueous phase (W_1) including 1 ml, 1.2 ml, 1.5 ml and 2 ml of distilled water to determine its effects on the final PCL particle prepared by double emulsion. These volumes were emulsified in 25% solution of polycaprolactone in DCM. It was found that the volume of the internal aqueous phase has no significant effect on the size of the final particle in double emulsion. By increasing the volume of inner phase there was insignificant decrease in size of microparticles with some variations (Fig. 6). Smallest particle size was achieved at 2 ml of inner aqueous phase. The smaller particle size at high volume of inner aqueous phase may be due to the factor that; the high polymer amount solution coagulates faster during the second emulsion and results in tighter structure due to chain entanglement [40]. So at high volume ratio of inner aqueous phase (W_1), polymer may form thin layer over water droplet and thus a low probability to coagulation into large particle.

3.1.6. Effect of outer aqueous phase volume

Four samples were prepared with different volumes of outer aqueous phase (Table 2) and its effect was evaluated on the final PCL particles prepared by double emulsion. It was found that by increasing the volume of outer aqueous phase (W_2) the size of the microparticles decreased slightly with some variations (Fig. 7). Larger particles were obtained at 50 ml and smallest particles were achieved at 100 ml volume of the outer aqueous phase in this study.

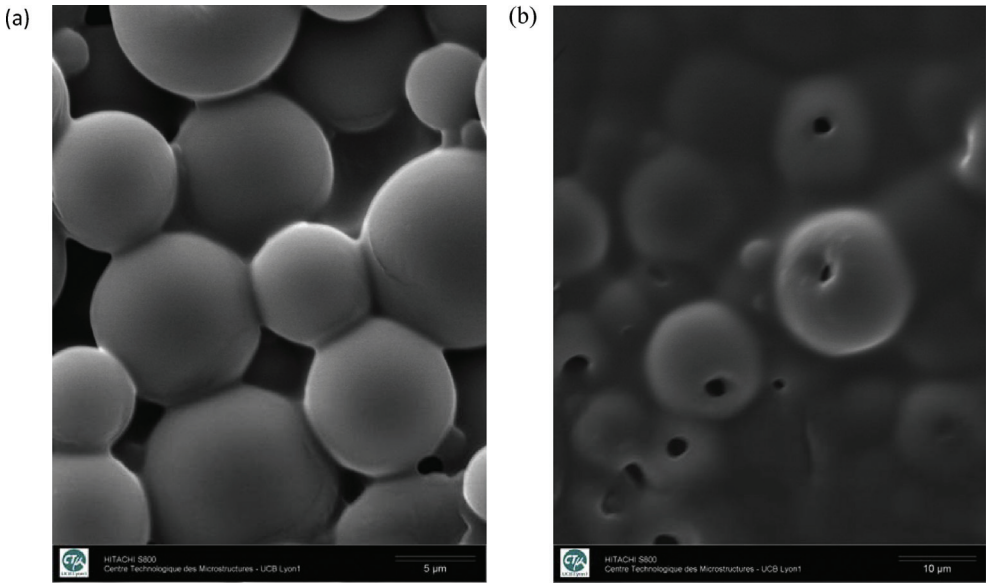

Fig. 11. (a) SEM image of PCL particles prepared by double emulsion like method. The scale bar represents 5 μm. (b) SEM image of PCL particles prepared by double emulsion like method. The scale bar represents 10 μm.

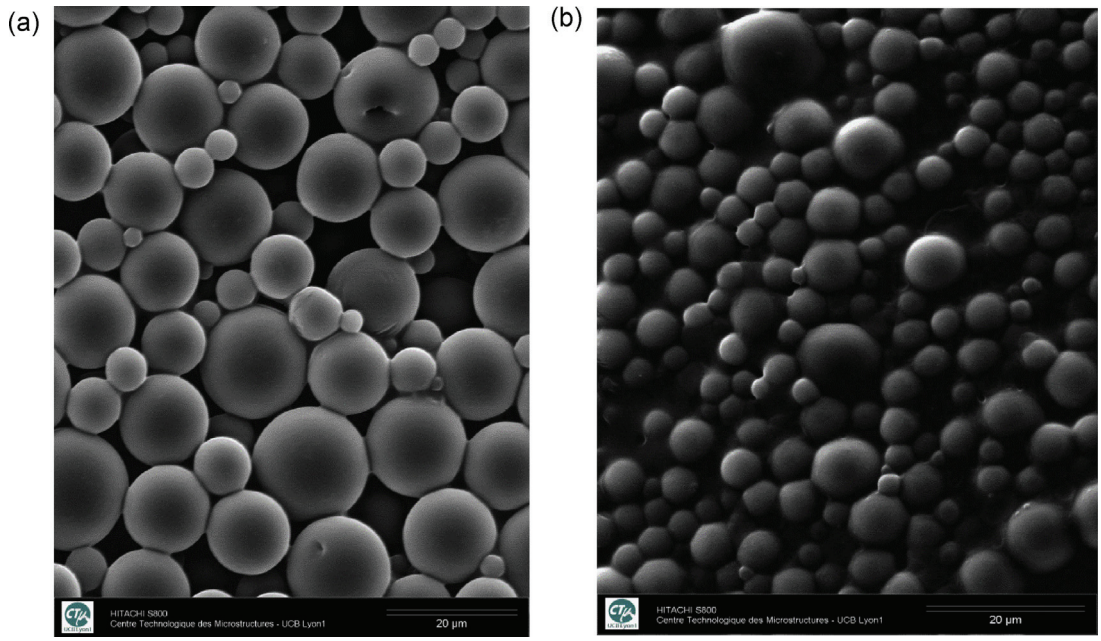

Fig. 12. (a) SEM image of PCL microparticles prepared using 3 g of PCL. (b) SEM image of PCL microparticles prepared using 1 g of PCL.

This may be due to decrease in the viscosity of the emulsion at high volume of outer phase, so there is efficient shearing force to reduce the particle size.

3.1.7. *Effect on particle size distribution*

Particle size distribution for different recipes was investigated using laser diffraction. Size distribution was found to be large and ranging from 0.4 to 40 μm revelling incontestably the polydispersity of the prepared dispersions. But for low polymer amount (i.e. 1 g) one single size distribution peak (0.4–20 μm) was observed (Fig. 8a). Whereas, above 1 g of PCL tow peaks were obtained as shown in Fig. 8b and c.

In case of PVA concentration, particle size distribution ranges from (0.4 to 40 μm) irrespective of PVA amount with the presence of two distributions as shown in (Fig. 8d and e).

3.2. *Zeta potential*

Actually, the value of zeta potential reflects the charge of particles surfaces and this value depends on three factors, the chemical nature of the polymer, the surfactant, and medium pH values [22].

In this study, zeta potential was measured for all samples at different pH i.e. pH 3, 5, 7, 9 and 11 (Table 3), and it was found that there was no significant change in the values of zeta potential of all samples (Fig. 9), which means that changing of PCL microparticles preparation conditions such as changes in polymer concentration, stabilizer amount, stirring time and speed, phase volume for 1st and 2nd emulsion have no significant effect on the zeta potential. In fact, the zeta potential was found in between +1 and −4 mV which can be considered in zero range reflecting the non charge character of the particles as already reported in the literature [46]. This low zeta potential can bi attributed to non-charge character of PCL.

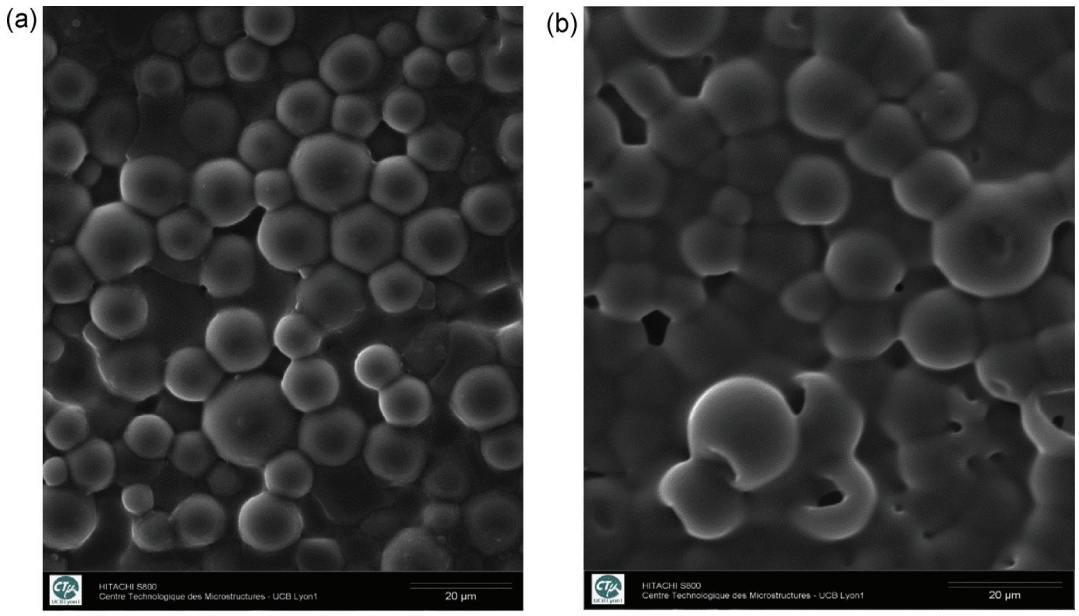

Fig. 13. (a) SEM image of PCL microparticles prepared in 0.5% PVA concentration. (b) SEM image of PCL microparticles prepared in 2% PVA concentration.

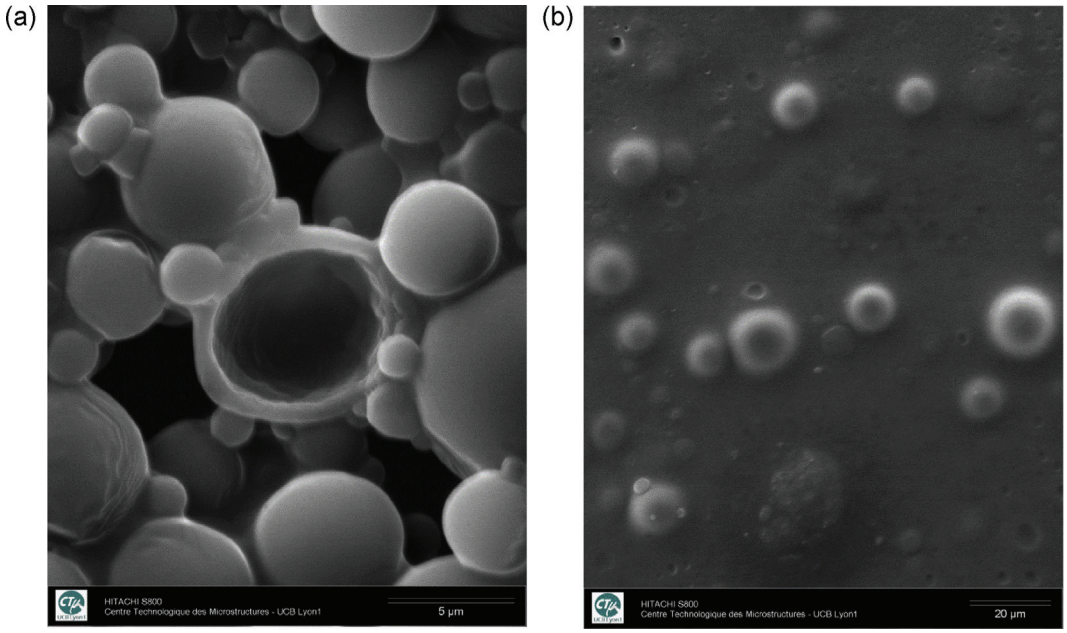

Fig. 14. (a) SEM image of PCL microparticles prepared under 4 min stirring. The scale bar represents 5 µm. (b) SEM image of PCL microparticles prepared under 6 min stirring.

3.3. Effects of different parameters on the morphology of the particles

The morphology of the PCL microparticles prepared by double emulsion technique was observed with help of Field Emission Scanning Electron Microscope (S-800 SEM Hitachi, Japan). Fig. 10a shows that the shape of the obtained microparticles can be assume to be spherical with smooth surface and unimodel size distribution Fig. 11(b). Compare to the results obtained from Laser Diffraction Particle Size Analyzer, the particles measure with SEM were slightly smaller this may be due to contraction induced by drying during evaporation of solvent. SEM images (Fig. 11a) show that some particle are observed to connected with each other, which may be due to surface tension of water acting on microparticles during drying which is in accordance to the results observed by Wu et al. [47]. These images show that all the particles are rounded shape. Some particles have pores on their surface, which may be due to evaporation of solvent during dying (Fig. 11b).

3.3.1. Polymer amount

The polymer amount is significant factor that affects the characteristic of microparticles prepared by double emulsion method [40].

In this work, we observed two samples prepared with polymer amount of 1 g and 3 g. From SEM images observations it was found that, the particles obtained with 3 g polymer (Fig. 12a) having

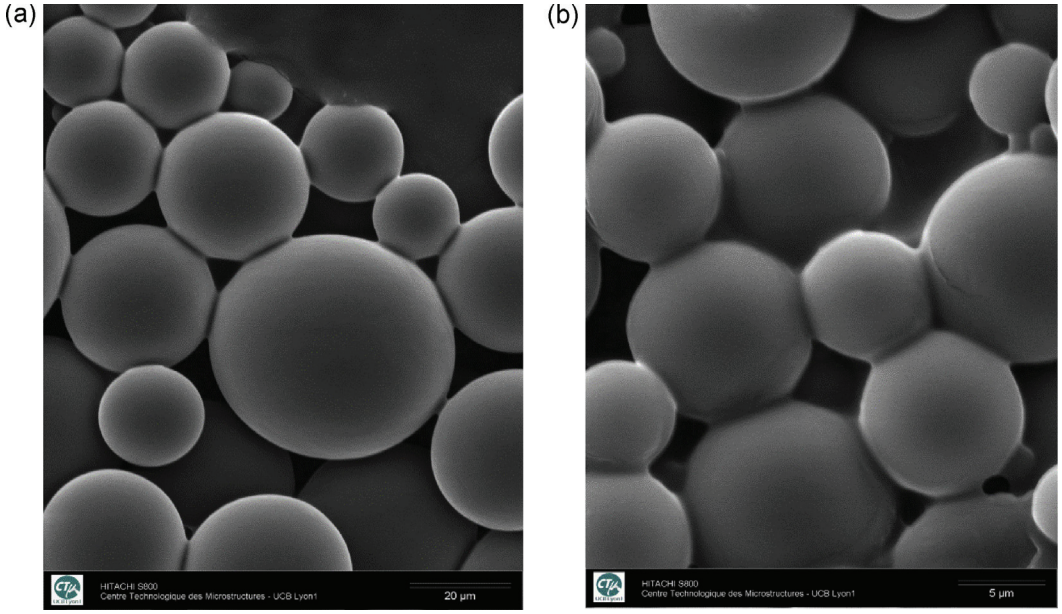

Fig. 15. (a) SEM image of PCL microparticles prepared under a stirring speed of 9500 rpm for the second emulsion. (b) SEM image of PCL microparticles prepared under a stirring speed of 21,500 rpm for the second emulsion. The scale bar represents 5 µm.

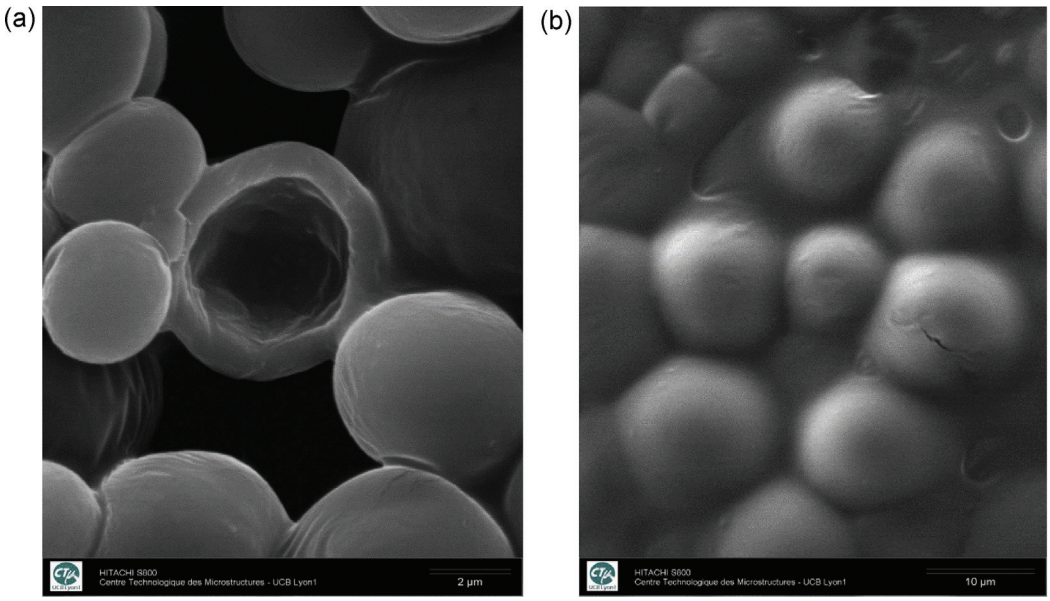

Fig. 16. (a) SEM image of PCL microparticles prepared with 50 ml of outer aqueous phase. The scale bar represents 2 µm. (b) SEM image of PCL microparticles prepared with 150 ml of outer aqueous phase. The scale bar represents 10 µm.

narrow size distribution and more regular shape as compare to particles obtained from 1 g polymer amount as shown in Fig. 12b.

3.3.2. *The influence of PVA concentration in the external water phase*

The significant influence of the concentration of PVA in the external water phase on the size of resultant particles is reported in many studies [48–50]. In this study, two samples were prepared with PVA concentration of 0.5% and 2% in the external water phase and its influence on the particle morphology was studied using SEM. From the SEM images it is evident that the particles prepared with 0.5% PVA has shown good morphology with regular rounded shape (Fig. 13a) as compare to particles prepared with 2% PVA, which were clumped together as show in Fig. 13b. This clumping may be due excessive PVA, which results in sticking of particles during drying.

3.3.3. *The influence of time of agitation*

The influence of agitation time for both first and second emulsion was studied. For 1st emulsion, particles were papered with agitation time of 4 min and 6 min. From the SEM images, it was found that agitation time of first emulsion has negligible effect on the size and morphology of particles.

In second emulsion the particles was prepared with the same agitation time i.e. 4 min and 6 min. When the samples of second emulsion were observed with SEM, it was found that the particles papered with 6 min agitation time has regular morphology (Fig. 14b) on the other hand the particles with 4 min agitation time have not well encapsulated the inner water phase and some

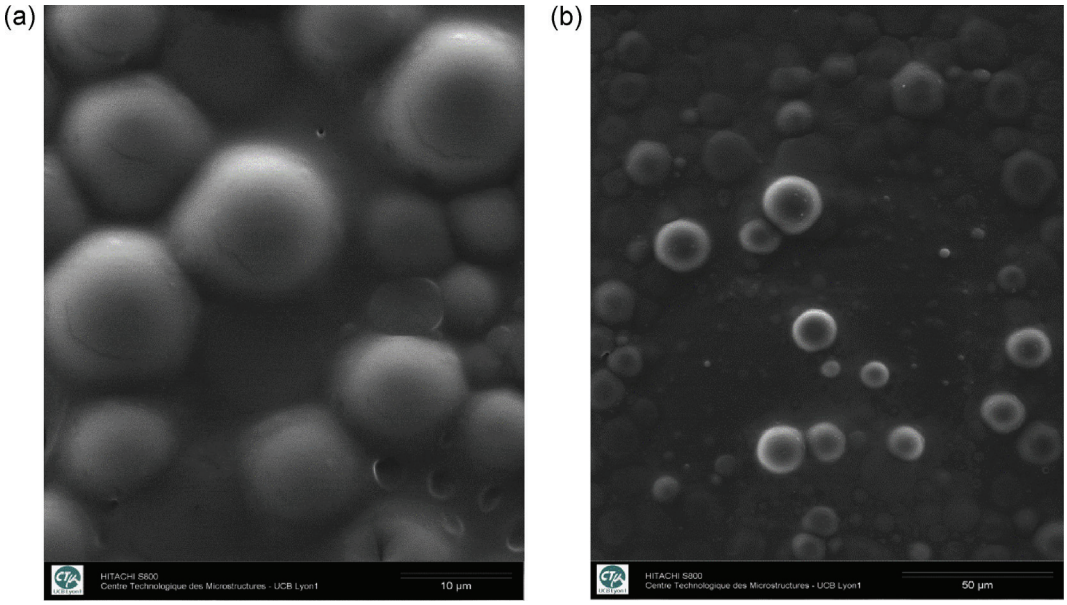

Fig. 17. (a) SEM image of PCL microparticles prepared using 1000 µl inner aqueous phase volume. The scale bar represents 10 µm. (b) SEM image of PCL microparticles prepared using 1500 µl inner aqueous phase volume. The scale bar represents 50 µm.

particles have cup shape morphology as shown in Fig. 14a. The homogeneity of particles size increased when the second emulsion was agitated for long period of time, these results in accordance with the results reported by Ayoub et al. [2].

3.3.4. The influence of stirring speed

The stirring speed is the vital parameter affecting the particle size because it supplies the necessary energy to disperse the dispersed phase into continuous phase, some researchers reported that, the second emulsion is preferred to prepare by avoiding sever mixing because it may destroy the first emulsion W_1/O prepared in the first step [34]. From SEM images, it was found that stirring speed of first emulsion has no significant influence on the particle shape and size. In case of second emulsion, SEM study established that the microparticles prepared with 21,500 rpm have narrow size distribution (Fig. 15b) as compare to particles prepared with 9500 rpm (Fig. 15a).

3.3.5. Effect of outer water-phase volume

The volume of external water phase is an important factor that plays vital role in determining the size, shape and size distribution of resultant particles.

From SEM observation, it was found that the particles prepared by using external water phase 50 ml, some particles obtained have cup shape morphology as shown in Fig. 16a. On the other hand the particles obtained with 150 ml external aqueous phase has regular rounded shape and narrow size distribution (Fig. 16b).

3.3.6. Influence of inner water phase (W_1) volume

The effect of this parameter was studied by comparing the SEM photographs of two samples, in first one the internal aqueous phase was1000 μl and in the second it was 1500 μl.

From the SEM images observation, it was found that the particle obtained with 1000 μl internal aqueous phase have cracks on their surfaces with spherical shape Fig. 17a, while the other particles which are prepared by using 1500 μl as internal aqueous phase have rounded shape and good morphology.

4. Conclusion

Multiple emulsions are important systems; consisting of two immiscible phases, this system is thermodynamically unstable so suitable emulsifying agent is added in order to stabilize it.

In this study the conditions and parameters which influencing properties especially particle size and morphology of double emulsion were well studied. Microparticles were prepared by using two-step emulsification solvent diffusion process. The effect of different parameters such as Stirring time (of 1st and 2nd step), stirring speed (of 1st and 2nd step), polymer amount, stabilizer concentration, phase volume of aqueous passes (inner and outer) on the partial size was investigated.

The main finding of this study was that:

There was no significant effect of stirring speed in double emulsion preparation during step first, but during step 2nd with an increase in stirring speed the particle size of emulsion decreased proportionally.

The time duration of stirring in first step of double emulsion has no significant impact on particle size while during 2nd step longer stirring time has led to smaller size particles.

The concentration of polycaprolactone (selected polymer for encapsulating) has a significant influence on the particles size i.e. particles size proportionately increased when the amount of PCL were increased. In case of stabilizer concentration it was concluded that at low concentration of PVA (stabilizer) smaller size particle can be achieved than at higher concentration.

The relative volumes of aqueous phases are also an important factor in double emulsion particle size evaluation; from this study it is established, that the phase volume of inner aqueous phase (W_1) has no significant effect on the particle size of double emulsion while on the other hand volume of outer aqueous phase (W_2) has a prominent effect on the final particle size.

From SEM images observation, it was found that the surface of obtained microparticles can be assumed to be spherical with smooth surface, having narrow size distribution. Compare to the results from Laser Diffraction Particles Size Analyses, the particles measure with SEM were slightly smaller in size this may be due to contraction induced by drying during evaporation of solvent.

In this work we study the effects of different parameters affecting the particle size of double emulsion, further evaluation study have to be performed in order to determine suitable amount of active ingredient such as proteins to be loaded in the inner aqueous phase (W_1) and also to study the release of active medicaments from these microparticles.

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III.2. Preliminary study of particles preparation via double emulsion using power ultrasound

General summary

Recently, polymer-based biodegradable particles got much attention in biomedical field due to their special properties and wide application in the drug delivery systems. Biodegradable particulate drug delivery has been widely studied mainly for parenteral, aerosol, oral and ocular applications. For the preparation of these particles, several methods have been developed but the most popular is emulsion solvent evaporation method and its modified version, the double emulsion solvent evaporation method. The main challenge of this method is the optimization of different process parameters that could be used for preparation of particles with appropriate size and size distribution. This study was performed with the aim, to investigate the effects of preparation conditions on the particles properties (particle size, zeta potential, morphology etc), prepared by double emulsion evaporation method using power ultrasound and to reduce the particle size compared to the previous systematic study. Two steps double emulsification method (W1/O/W2) was used, in the first step, distilled water was emulsified with organic phase (polycaprolactone dissolved in dichloromethane) via sonication to form first emulsion (W1/O), followed by addition of first emulsion in the outer aqueous phase (Polyvinyl alcohol solution) to form double emulsion. And subsequent evaporation of organic solvent via rotary evaporator from dispersed phase resulted in particulate suspension. Polycaprolactone was used as polymer and dichloromethane as organic solvent in the first step while, polyvinyl alcohol was used as stabilizer in the second step.

The effects of parameters studied were included; ultrasound exposure time in the first and the second step of emulsification, ultrasonic amplitude, stabilizer concentration, polymer amount and outer aqueous phase volume. It has been demonstrated that ultrasound emulsification is an efficient method to obtained nanoparticles via double emulsion solvent evaporation technique. Sonication amplitude has a significant effect on particles size and morphology. In second step, smaller size particles were obtained at higher amplitude of ultrasound. It was reported that, modification in ultrasound exposure time has no significant impact on mean particle size in the first step of emulsification, while during the 2nd step an increased ultrasound exposure time has led to smaller size particles. In case of polymer amount, small size (235 nm) nanoparticles were obtained when 1 g of polycaprolactone was used as compared to 5g of PCL (748 nm). It was concluded that presence of PVA (stabilizer) is compulsory for NPs preparation by this process (in absence of stabilizer rapid phase separation were observed), and the particle size decreases by

increasing PVA concentration from 0.05% to 0.2% and beyond this value there was no significant effect on mean particle size.

Moreover, it was shown that, the particle size decreases significantly with an increase of outer aqueous phase volume from 50 ml to 150 ml, and beyond this value, no marked effect were observed. From SEM images observation, it was found that the surface of obtained nanoparticles can be assumed to be spherical with smooth surface, having broad size distribution but results are comparable with other high speed homogenizers. The change in preparation conditions have no effect on the zeta potential of polycaprolactone nanoparticle prepared by this technique. This may be due to non-charged nature of polycaprolactone.

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ORIGINAL CONTRIBUTION

Preparation of biodegradable PCL particles via double emulsion evaporation method using ultrasound technique

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Abstract Polymeric nanoparticles have attracted growing attention because of their unique properties and extensive application. In this study, polycaprolactone (PCL) nanoparticles were prepared via double emulsion solvent evaporation-like process using power ultrasound, and the effects of various process parameters on particle size, zeta potential, and morphology were investigated and optimized. Nanoparticles (NPs) were prepared by two-step emulsification process. In the first step, the inner aqueous phase (W_1) was homogenized with organic phase (PCL in dichloromethane) to obtain primary emulsion. In the second step, the primary emulsion was emulsified with outer aqueous phase (W_2) containing polyvinyl alcohol (PVA) as stabilizer using power ultrasound, followed by evaporation of solvent which resulted in a particulate suspension at the end. Effects of various parameters like ultrasound exposure time and amplitude, outer aqueous phase volume, PVA concentration, and PCL content were investigated. It has been shown that, by increasing ultrasound exposure time, amplitude, and outer aqueous phase volume, the particle size decreases. Additionally, particle size was also related to amount of PCL and PVA concentration. Spherical NPs with smooth surfaces were observed by scanning electron microscopy (SEM).

Keywords Double emulsion process · Nanoparticles · Colloidal properties · Parameters · Size · Ultrasound

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Introduction

Recently, nanoparticulate carriers are getting more attraction due to their potential application in targeted drug delivery systems, [1] food technologies, and cosmetics [2–4]. There has been extensive research in processes like double emulsion, self-assembly, and phase separation for fabrication of colloidal polymeric nanoparticles with unique shapes and properties [5]. The preparation method mostly depends on the nature of drug (hydrophilic or hydrophobic) to be encapsulated [6]. Double emulsion technique is an appropriate method often used for encapsulation of hydrophilic molecules, in which first, aqueous phase is dispersed in nonmiscible organic solvent containing polymer to form primary emulsion (W_1/O), followed by the homogenization of primary emulsion into outer aqueous phase containing emulsifier to get the double emulsion using high-shear homogenizer or sonotrode. Subsequently, the evaporation of organic solvent from emulsion leads to particulate suspension at the end [7]. Pharmaceutical researchers are highly interested in fabricating a drug delivery system that enhances the drug efficiency, bioavailability of poorly soluble drugs and to minimize the undesirable effects [8, 9]. Encapsulation of active moiety is one of the vital techniques to protect the drug from the harsh environment of the stomach and degradation. Especially for labile moiety like proteins and DNA, it is essential to avoid their denaturation during their transport to the site of action. In drug delivery systems, colloidal carriers like nanoparticles are becoming more important due to their smaller size, which enables them to permeate through biological membranes [10–12]. The first paper on double emulsion dates back 89 years [13]. It can be identified as complex polydispersed systems and can be classified into two types: water-oil-water emulsion and oil-water-oil emulsion. They are usually prepared by two-step emulsification process [14, 15]. Generally, for emulsion preparation, we need oily phase, aqueous phase, surfactant, and

appropriate energy. The type of the resulting emulsion is determined primarily by type of surfactant used. In emulsions, smaller size droplets are more stable against creaming, coalescence, and flocculation [16]. Thus, droplet size plays a vital role in emulsion stability [17, 18].

Double emulsion process is very versatile which can be used for fabrication of various kinds of polymeric particles including nanoparticles with hollow structures [19]. The emulsion droplets can be used as templates for further processing to form core-shell nanostructures, by emulsifying the polymer-containing dispersed phase into outer aqueous phase and then removing the organic solvent via evaporation or diffusion, leaving polymeric dispersion [20, 21]. Several authors have prepared nanoparticles by double emulsion solvent evaporation techniques from different polymers like poly(lactic acid) [22], polycaprolatone (PCL), and poly(D,L-lactic-co-glycolic acid) [23, 24]. PCL is a biodegradable polymer with low glass transition temperature and melting point, and the polymer metabolites are eliminated from the body by innate metabolic process [25]. Due to biodegradable, biocompatible, and nontoxic nature of PCL, it is extensively studied for control drug delivery system in several formulations including nanoparticles, implants, nanofibers, microspheres, etc. Its compatibility with wide range of drug and its slow degradation to release drug for extended period of time (month-years) makes it an appropriate candidate for controlled drug delivery systems. Moreover, it allows the modification of its physicochemical and mechanical properties by copolymerization, which in turn affect all other properties of PCL such as solubility, ionic property, and degradation pattern [26]. Poly vinyl alcohol (PVA) is one of the most commonly used and commercially available polymer stabilizers [27]. It is a well-known hydrophilic, biocompatible polymer and has good mechanical strength, low fouling potential, and long-term temperature and pH stability. These properties of PVA make it appropriate stabilizer for nanoparticle preparation for medical and pharmaceutical applications [28].

Ultrasound emulsification is an efficient method to obtain a finely dispersed emulsion; typical results are comparable with those of the best high-pressure homogenizers [29]. The advantages of ultrasound include lower energy consumption, production of more homogeneous emulsion, with smaller droplet size and more stable emulsion compared to a mechanical homogenization with use of less surfactant [30, 31]. However, the mechanism of emulsification using ultrasound-based technique is not fully known, but it is thought that emulsification might occur by "transient" acoustic cavitation produced by ultrasound's horn. It was proposed that there are two steps in acoustic emulsification: First, interfacial instability at oil-water interface is caused by low-frequency acoustic waves which are followed by formation of transient cavitation bubbles in the second step [32]. Cavitation is the formation and collapse of vapor cavities in the liquid, generated by the mechanical vibration of sonicator probe. It is an important phenomenon that occurs during transmission of acoustic power into liquid system. When liquid is exposed to very low ultrasonic power and the power is gradually increased, a point is reached at which the transmitted acoustic energy is sufficient to cause cavitation in the fluid, this minimum energy needed to form cavitation is termed as cavitation threshold. Majority of sonochemical effects including emulsification occurs only at power above the cavitation threshold [18, 33]. Several parameters affect the emulsification process by ultrasound including hydrostatic pressure, viscosity of continuous phase [34], oil/ water ratio, surfactant concentration, position of ultrasonic horn at oil-water interface, and ultrasonic power and exposure time [30, 35–37].

With the progress in new emulsification techniques used for encapsulation and their application in drug delivery system, it is essential to understand the mechanism of emulsification and process parameters affecting the emulsification process. Most of the published work in emulsion field is dealing with pure emulsions consisting of water, oil, and emulsifier, while there has been very limited work to produce emulsion with subsequent encapsulation of submicron particles, in which another component is involved called wall material (biopolymer). The objective of the present work was to investigate the most influential process parameters in preparation of optimal NPs by double emulsion solvent evaporation using power ultrasound. These parameters were the polymer content, concentration of stabilizer (PVA), outer aqueous phase volume (W_2), sonication time, and amplitude. The influence of these parameters on the particle's average hydrodynamic size, zeta potential, and morphology was studied.

Experimental section

Materials

Polycaprolactone (PCL) (Mw=14000 g/mol), polyvinyl alcohol (PVA) (Mowiol® 4–88, Mw=31000 g/mol), and dichloromethane (CH_2Cl_2, Mw=84.94 g/mol) were obtained from Sigma-Aldrich, Germany, and used as such. Water was deionized using Aquadem® (Veolia Water, France). Ultrasonic homogenizer system is "CY-500" ivymen® (500 W, 20 kHz) from SELECTA GROUP, Switzerland. Analytical balance (Acculab ALC-110.4) was supplied by Sartorius group, Germany. S-800 FEG Scanning Electron Microscope was obtained from Hitachi, Japan. Zetasizer 3000 HSa and Zetasizer Nano-ZS were supplied by Malvern, UK.

Methods

Double emulsion solvent evaporation technique

The double emulsion solvent evaporation is a widely used technique for preparation of nanoparticles [38]. This method was previously used for the preparation of microparticles [39]. In this technique, the primary emulsion (W_1/O) is prepared by homogenization of inner aqueous phase (W_1) with organic phase (polymer solution). Then, the primary emulsion is added to external aqueous phase and homogenizes to form nanoemulsion. Nanoemulsion formation is followed by evaporation of organic solvent from the dispersed phase leading to a point of insolubility and precipitation of the polymer encapsulating the active material. The outer aqueous phase acts as dispersion medium. The solvent may be evaporated by simple stirring at ambient temperature or under reduced pressure by rotary evaporator depending upon the nature of organic solvent.

Preparation of PVA solution in water

PVA solution (0.5 %) was prepared in order to be used as outer aqueous phase (W_2), by taking 5 g of PVA in 1000-ml flask, and sufficient amount of deionized water was added to make up the volume. PVA was dissolved under magnetic stirring at 60 °C for 40 min, which resulted in a clear PVA solution.

Preparation of nanoparticles

Nanoparticles were prepared by double emulsion solvent evaporation-like process using power ultrasound. Double emulsion was prepared by two-step emulsification process.

In the first step, in order to make the primary emulsion (W_1/O), 3 g of PCL was dissolved in 12 ml of dichloromethane (DCM) properly to form a clear solution (oil phase), and then, 1.5 ml of deionized water (W_1) was dispersed in PCL solution. This mixture was homogenized properly using ultrasonic homogenizer "CY-500" ivymen® at a 70 % amplitude for 5 min.

In the second step, the primary emulsion (W_1/O) was dispersed in the outer aqueous phase (W_2) containing 0.5 % PVA as stabilizer in 250-ml glass beaker. This mixture was homogenized via ultrasonic homogenizers at specific amplitude for specific time (Table 1), which produced double emulsion ($W_1/O/W_2$). The ultrasonic horn was positioned 2 mm above the oil-water interface in the system and was kept constant for all experiments. Afterword, the organic solvent evaporation from dispersed droplets via rotary evaporator has led to solidified PCL nanoparticles. These dispersed particles were then recovered by centrifugation at 10,000 rpm for 10 min and washed three times with deionized water properly. The ultrasonic transducer (homogenizer) used was of 500 W power and 20-kHz frequency, consisting of titanium alloy probe (5.6-mm diameter and 60-mm height). The above-mentioned conditions were to prepare reference recipe, and in all other experiments, only one parameter was changed each time, while the rest of the parameters were kept fixed. The double emulsion solvent evaporation process used to prepare particles is schematically described (Fig. 1).

Reference emulsion composition

Specific values of different parameters were used to formulate the reference emulsion as shown (Table 1). Afterward, several sets of experiments were performed to investigate the effects of different preparation conditions on the characteristics of the nanoparticles prepared via double emulsification solvent evaporation process by changing only one parameter and keeping all other conditions constant. For example, to study the parameter "polymer amount effect," five samples were prepared with different amount of PCL, i.e., 1, 2, 3, 4, and 5 g while keeping all other conditions constant as given in table (Table 1).

Hydrodynamic size measurement

After preparation, the average hydrodynamic particles size was determined by Zetasizer HSa 3000, Malvern. The mean particle size was the average of three independent measurments. Each sample was prepared by adding one drop of particulate dispersion in about 1.5 ml of deionized water in quartz cell, and then, the cell was placed in zetasizer for analysis. Mean particle size was determined for each preparation.

Zeta potential

The zeta potential of different nanoparticle preparation was determined by using Nano-ZS Malvern. Measurements were performed at different pH values (pH 3±0.2, pH 5±0.2, pH 7 ±0.2, pH 9±0.2, pH 11±0.2) using Malvern Auto-titrator MPT-2 in aqueous dispersant (10^{-3} M NaCl) at 25 °C.

Particle morphology

Scanning electron microscopy (SEM) was performed with Hitachi S800 FEG microscope at the "Centre Technologique des Microstructures" (CTμ) at the University of Lyon (Villeurbanne, France). A drop of diluted aqueous suspension of nanoparticles was deposited on a flat steel holder and dried at room temperature. The sample was finally coated under vacuum by cathodic sputtering with platinum (5 nm). The samples were observed by SEM under an accelerating voltage of 15 kV. Before deposition on steel holder, all samples of particle were centrifuged at 1000 rpm for 10 min and washed

Table 1 Reference emulsion composition used for preparation of nanoparticle via double emulsion evaporation process

Primary emulsion's parameters (first step)				Double emulsion's parameters (second step)			
Amount of PCL (g)	Inner aqueous phase W_1 (ml)	Ultrasound's amplitude (%)	Sonication time (min)	Concentration of PVA (%)	Ultrasound's amplitude (%)	Sonication time (min)	Outer aqueous phase W_2 (ml)
3	1.5	70	5	0.5	70	8	150

three times with deionized water in order to remove the excess of PVA.

Results and discussion

Effect of different parameters on the mean particle size obtained

The effects of different process parameters (for given conditions) were first investigated in order to point out the relationship between the used conditions, the colloidal stability, and the colloidal properties of the formed dispersion, and also the relationship between different parameters (energy, time, volume, polymer, stabilizer) and the final hydrodynamic particle size.

Effect of ultrasound exposure time

Ultrasound exposure time is vital parameter affecting ultrasonic emulsification process [36]. Exposure time effects were investigated for both steps of emulsification process in preparation of particles. During investigation of the ultrasonic exposure time for the first step of emulsion, the ultrasonic exposure time for the second was 8 min and constant, whereas, during investigation of the ultrasonic exposure time for the second emulsion, the exposure time for the first was constant and equal to 5 min.

In the first step, the inner aqueous phase (W_1) was emulsified with organic phase (PCL and DCM). Five samples of particle dispersions were prepared with different ultrasonic exposure time, i.e., 2, 4, 6, 8, and 10 min. The variation in mean particle size as a function of exposure time (min) is presented (Fig. 2), which showed that there was no significant variation in mean hydrodynamic size when exposure time was increased from 2 to 10 min during the first step of emulsification process.

In the second step, several experiments were performed under sonication of 2, 4, 6, 8, and 10 min using 70 % duty cycle. It was found that, with increase in ultrasound exposure time, the hydrodynamic particle size was decreased gradually as shown in Fig. 2. This may be due to increasing energy (energy input) with elongated ultrasound exposure time, which causes more droplet fragmentation and consequently decreases in final particle size. These results are in agreement with the reported tendency by Jafari et al. [36]. It was observed that for efficient homogenization, the tip of ultrasonic horn

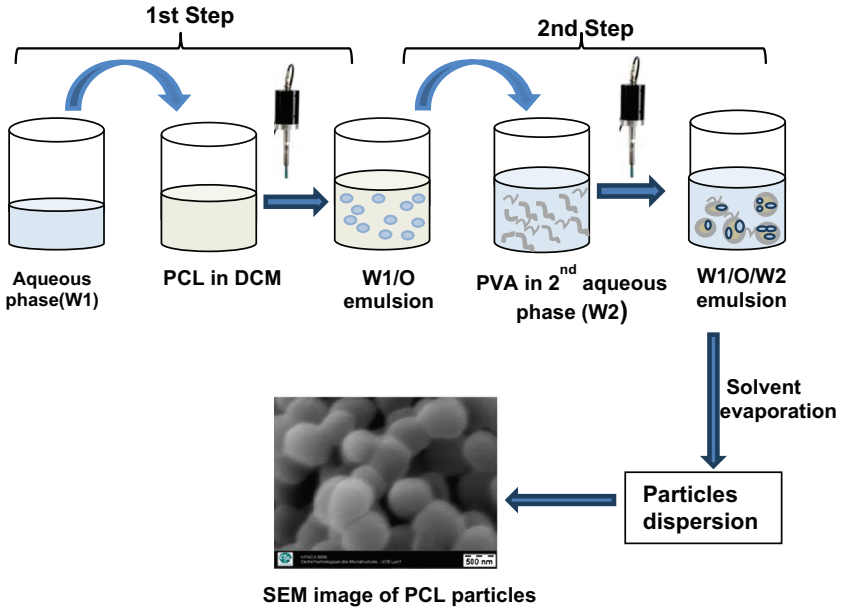

Fig. 1 Schematic illustration of two-step double emulsion process for preparation of nanoparticles using ultrasonic homogenizer. **a** The first step: homogenization of water (W_1) with PCL solution in DCM. **b** The second step: dispersion of first emulsion (W_1/O) in to outer aqueous phase (W_2). Subsequently, evaporation of solvent from W_1/O/W_2 results in particulate dispersion. SEM image scale bare represents 500 nm

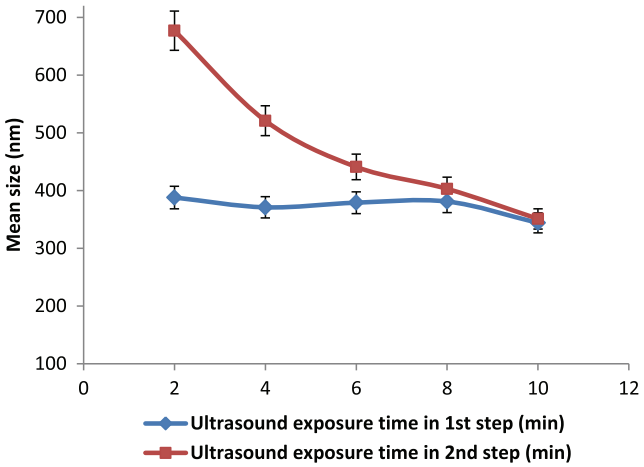

Fig. 2 Hydrodynamic particle size as a function of the ultrasonic exposure time. During the investigation of the ultrasonic exposure time for the first emulsion, the ultrasonic exposure time for the second was 8 min and constant, whereas in investigation of the ultrasonic exposure time for the second emulsion, the exposure time for the first was constant and equal to 5 min

should be at oil-water interface or few millimeters above it, which supports Cucheval et al. findings [35]. With an increase in ultrasound exposure time, a corresponding decrease in the average particle size during the second step and no significant influence on particle size during the first step was observed. This could be due to fact that, in the first step, there is only formation of unstable aqueous droplets, but no polymer particle solidification occurs; thus, the final particle size may not be influenced by the first step sonication time. However, in the second step, the diffusion of organic solvent from dispersed primary emulsion droplets results in solidification of polymer particle. As the solidified particle formation occurs during the second step only, that is why, the second step may be considered as the size determining step.

Effect of ultrasonic amplitude (second step)

During ultrasonic emulsification, cavitations occur when amplitude of applied sound source reaches a certain minimum value, called cavitation threshold [40]. If the amplitude is below threshold value, then cavitation and emulsification would not take place [41]. In the second step of emulsification, several particle preparations were made using different amplitude of ultrasound. Initially, 50 % amplitude was used for emulsification of primary emulsion with outer aqueous phase, but no emulsification occurred at this value. This may be due to insufficient energy transmission to the system, required to induce cavitation. Afterword, the amplitude was increased to 60, 70, 80, and 90 % in the next four recipes, which led to emulsification process and colloidal particulate dispersion. The average size of final particles with 60, 70, 80, and 90 %

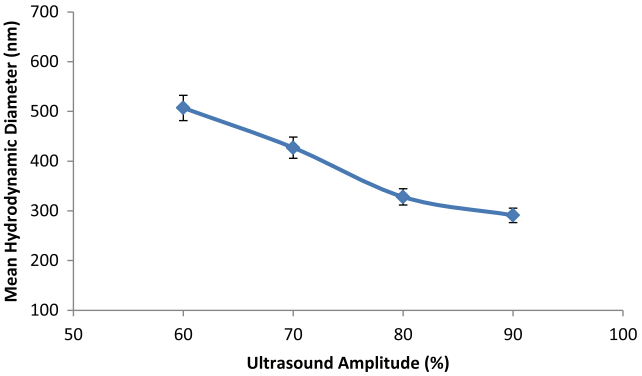

Fig. 3 Variations of mean particle size as a function of the ultrasound amplitude (%). All parameters were kept constant except ultrasound amplitude. Ultrasound amplitude was changed to 60, 70, 80, and 90 % in different colloidal preparations

amplitude was found to be 507, 427, 328, and 291 nm, respectively, as shown (Fig. 3).

This trend showed that by increasing amplitude of ultrasound homogenizer, there was reduction in mean size of the dispersion. This is probably due to high-energy dissipation in the system at high amplitude which results in deformation and break up of big droplets into small ones [35, 40].

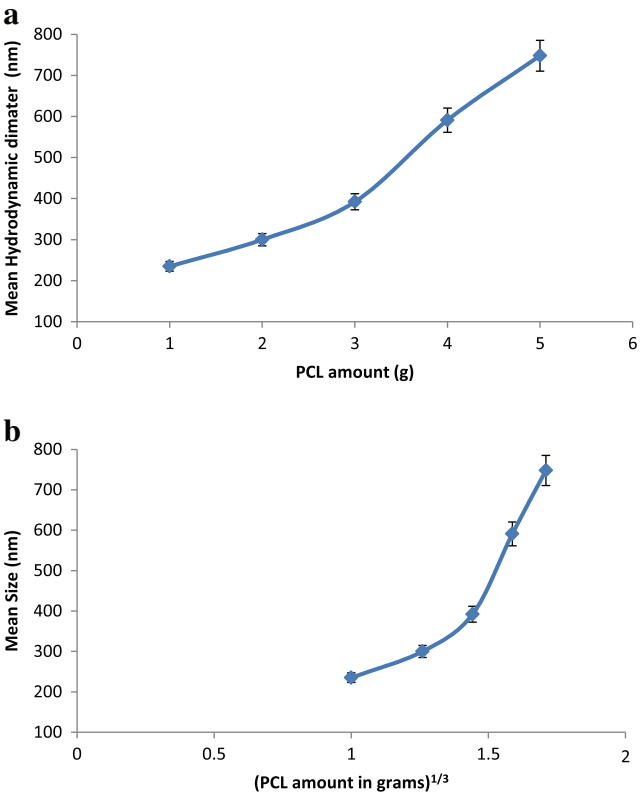

Fig. 4 **a** Effect of polymer content on the average hydrodynamic particle size. Only polymer amount was changed in different preparations, which was 1, 2, 3, 4, and 5 g of PCL, while all other parameters like ultrasound exposure time, amplitude, phase volume etc., were fixed. **b** Average hydrodynamic diameter of particle (nm) versus (PLC content in grams)$^{1/3}$

Table 2 Different parameters studied with their modified values used in various experiments, and the obtained average particles sizes are tabulated

Parameters studied	Changed value	Average particles size (nm)
Ultrasound exposure time in the 1st step (min)	2	388
	4	371
	6	379
	8	381
	10	344
Ultrasound exposure time in the 2nd step (min)	2	677
	4	521
	6	441
	8	403
	10	351
Ultrasound Amplitude (%)	60	507
	70	427
	80	328
	90	291
PCL amount (gr)	1	235
	2	300
	3	392
	4	591
	5	748
Outer aqueous phase volume (W_2)	50	521
	75	452
	100	441
	150	378
	200	375
	250	381
PVA concentration (%)	0.05	1456
	0.1	660
	0.2	392
	0.5	385
	1	376
	2	354
	3	364

Each time, only one parameter was changed, e.g., 1, 2, 3, 4, and 5 g of PCL were used during study of "PCL amount" effects while other parameters were kept fixed as given in Table 1

Effect of polymer amount

The polymer amount may be a vital factor influencing the particle's characteristics like drug encapsulation efficacy, particle size, size distribution, and morphology. In this study, PCL was used as polymer in different amounts (1, 2, 3, 4, and 5 g). Minimum size was observed (Fig. 4a) when small amounts (1 g) of PCL were used, whereas, when polymer amount was increased in different samples, consequently large particle sizes were obtained. This is in agreement with results

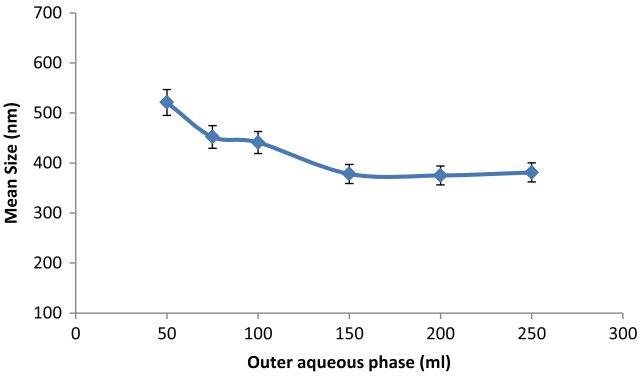

Fig. 5 Hydrodynamic particle size versus outer aqueous phase volume (W_2). Only outer aqueous phase volume was changed in different samples (i.e., 50, 75, 100, 150, 200, and 250 ml), and all other conditions were fixed

reported by Lamprecht et al. [24]. They stated that an increase in polymer amount leads to an increase viscosity of primary emulsion, thus results in less efficient reduction of particle size during the second step of emulsification process.

In order to know the real effect of polymer amount on the final particle size and number of particles (N_p), theoretical relationship between the particle size (d) and polymer amount (M) and the dispersion density (ρ) is expressed as follows:

$$d/2 = \left(3/4\pi\rho N_p\right)^{1/3} * (M)^{1/3} \quad (1)$$

According to this basic relationship, if the number of particle (N_p) is constant in the investigated polymer amount range, then the slope of hydrodynamic diameter particles versus and $(M)^{1/3}$ will be linear. But, in our case, it seems that the slope increases with the increasing amount of polymer in the formulation, as illustrated (Fig. 4b). This increase in the slope shows that by increasing polymer amount, the number of obtained particles also increases. Such increase in the number and in the size of particles can be attributed to the possible aggregation of unstable particles enhanced by increasing the solid content (polymer amount) and also by low stabilizing efficiency of PVA.

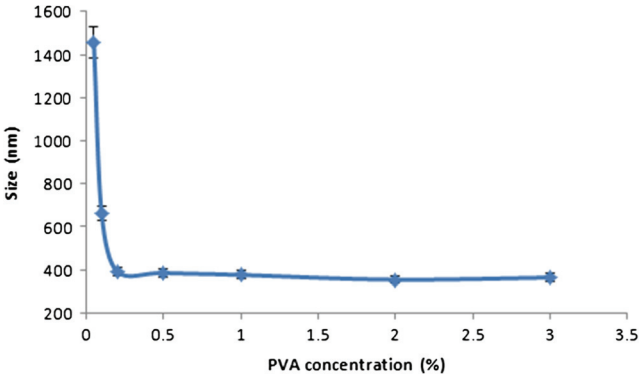

Fig. 6 Variations of mean particle size as a function of poly vinyl alcohol concentration (%). The concentrations of PVA used were 0.05, 0.1, 0.2, 0.5, 1, 2, and 3 % in different preparations

Table 3 Zeta potential of polycaprolactone particle dispersions at different pH values

Parameters studied	Zeta potential (mV) at different pH				
	pH=3	pH=5	pH=7	pH=9	pH=11
Ultrasound exposure time in the 1st step (8 min)	−0.34	−0.57	−0.71	−1.00	−1.56
Ultrasound exposure time in the 2nd step (10 min)	−0.47	−0.73	−0.75	−0.91	−0.84
Ultrasound amplitude (80 %)	−0.67	−0.96	−0.97	−1.4	−1.34
PCL amount (3 gr)	−0.68	−0.66	−0.84	−1.56	−1.12
Outer aqueous phase (200 W_2)	−0.32	−0.71	−0.74	−0.86	−0.84
PVA (1 %)	−0.12	−0.59	−1.00	−1.40	−0.68

Effect of outer aqueous phase volume

Outer aqueous phase volume is an important parameter affecting the particles and the dispersion properties. Six samples with different volume of outer phase volume, i.e., 50, 75, 100, 150, 200, and 250 ml (Table 2), were prepared, and its effect on particle's mean size and morphology was studied. It was observed that, when the volume of outer aqueous phase (W_2) increased from 50 to 150 ml, there was a significant decrease in mean particle size. But, beyond 150 ml (i.e., 150 to 250 ml), there was no significant variation in particle size as shown in figure (Fig. 5). At constant PCL concentration, this increase in particle size at lower volume of outer aqueous phase can be attributed to increase in viscosity. The higher the viscosity, the higher is the attractive forces between the molecules, and so, higher threshold intensity of ultrasound is required for onset of cavitation [29]. In addition, by decreasing dispersant phase volume, the probability of collision among particles increases consequently; coalescence, at a constant amount of PCL amount, is more probable.

Effects of stabilizer concentration

The addition of suitable stabilizer plays a key role in liquid-liquid dispersion. The concentration and type of stabilizer affect the colloidal stability of the prepared dispersion. The colloidal stability of a given dispersion is very important because during the evaporation of solvent, the volume of emulsion can decrease which in turn increases its viscosity. This may affect the final size of the droplet and may result in the coalescence and aggregation of the droplets during solvent evaporation (i.e., before reaching rigid-like polymer particles) [42, 43].

In this study, seven samples were prepared with different concentration of PVA in the outer aqueous phase (Table 2), and average hydrodynamic particle size was measured for each sample. It was found that initially, an increase in PVA concentration (0.05, 0.1, and 0.2 %) has led to rapid decrease in particle size as illustrated (Fig. 6). This result was found to be in agreement with the results reported by Zambaux et al. [22], although further increase in the concentration of PVA beyond 0.2 % has no significant effect on particle size. The

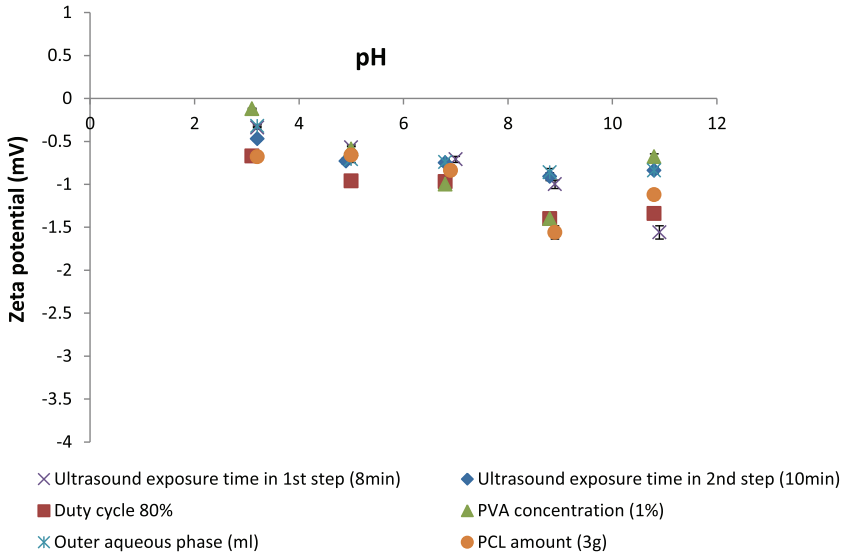

Fig. 7 Zeta potential (mV) of prepared particles (after DCM removal) versus pH and in 1 mM NaCl. Zeta potential was measured at pH 3, pH 5, pH 7, pH 9, and pH 11

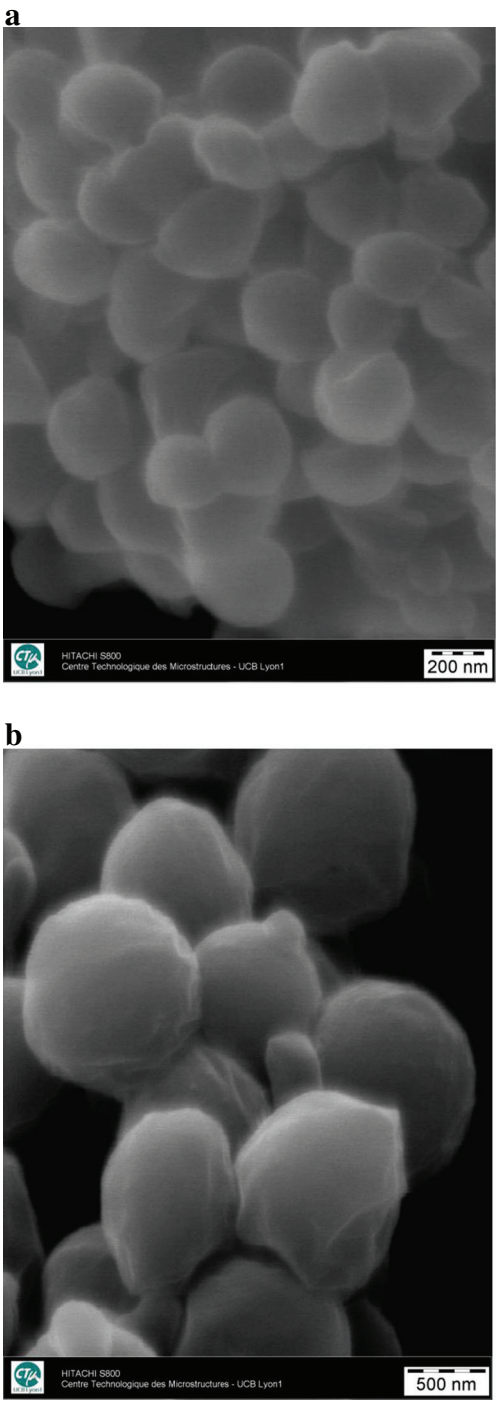

Fig. 8 **a** SEM image of PCL nanoparticles using 1 g of polycaprolactone. **b** SEM image of PCL nanoparticles using 3 g of polycaprolactone

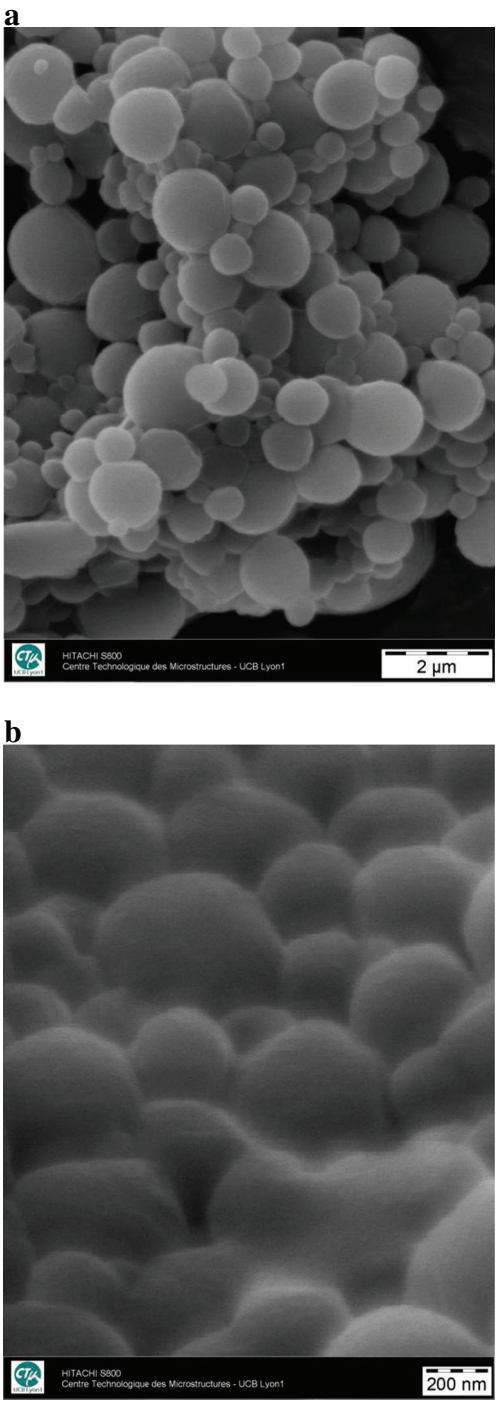

Fig. 9 **a** Nanoparticles prepared under 60 % ultrasound amplitude in the second step of emulsification process. **b** Nanoparticles prepared under 70 % ultrasound amplitude in the second step of emulsification process

increase in PVA concentration enhances the surface coating of the formed dispersion and also enhances the depletion colloidal stability of the formed droplets before solvent evaporation, which leads to smaller emulsion droplets and consequently smaller rigid particles after solvent evaporation [24]. Moreover, one sample was prepared without stabilizer (PVA) in outer aqueous phase (W2), but in this case, no homogeneous oil-water dispersion occurred, and rapid phase separation was observed.

Zeta potential

The zeta potential reflects the charge on the particle surfaces, and it depends on factors like the chemical nature of the

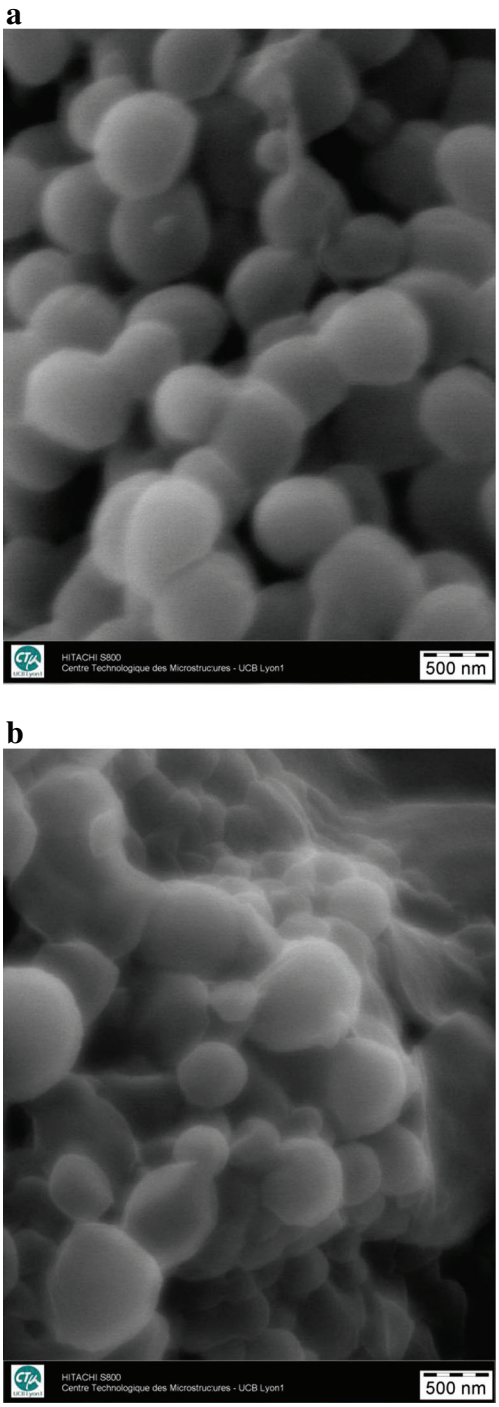

Fig. 10 **a** SEM image of nanoparticle prepared with 0.05 % PVA in outer aqueous phase. **b** SEM image of nanoparticle prepared with 0.5 % PVA in outer aqueous phase

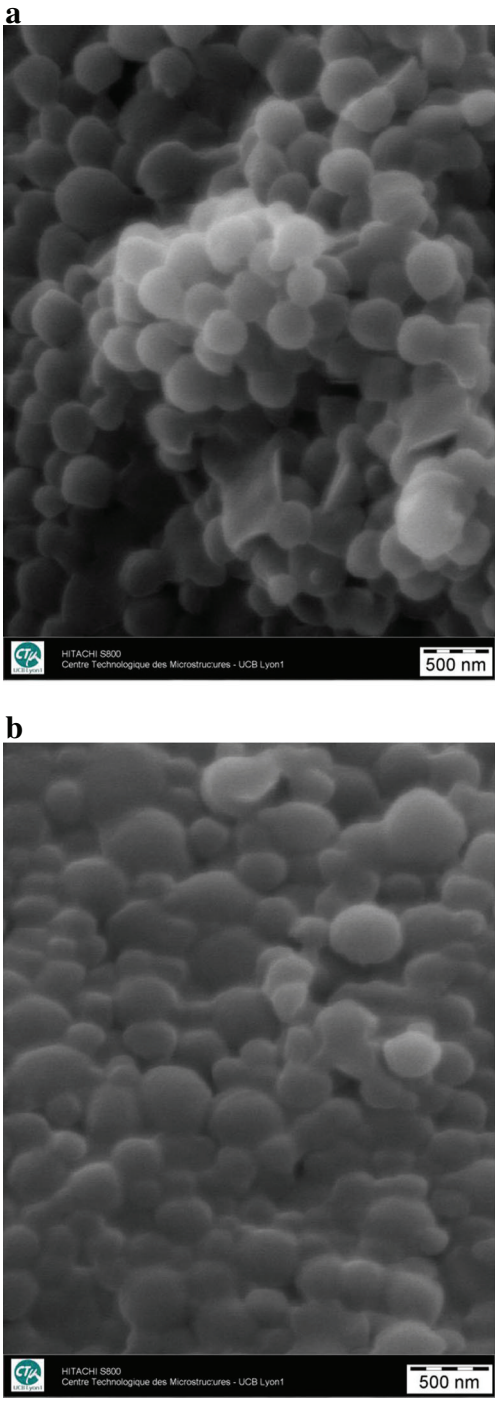

Fig. 11 **a** SEM image of PCL nanoparticles with 6-min exposure time during the first step of emulsification. **b** SEM image of PCL nanoparticles with 10-min exposure time during the first step of emulsification

polymer, the used stabilizer, and the pH values of the dispersant medium (22). The zeta potential of six samples was measured at different pH values (i.e., 3, 5, 7, 9, and 11). It was investigated in order to point out the relationship between the used conditions and the colloidal stability of the formed dispersion. In this study, the zeta potential was measured as a function of pH (Table 3), and it was observed that the pH has no significant effect on zeta potential of PCL particle prepared at different conditions (Fig. 7). These results reveal that modification in process parameter like ultrasound exposure time and amplitude, PVA concentration, PCL amount,

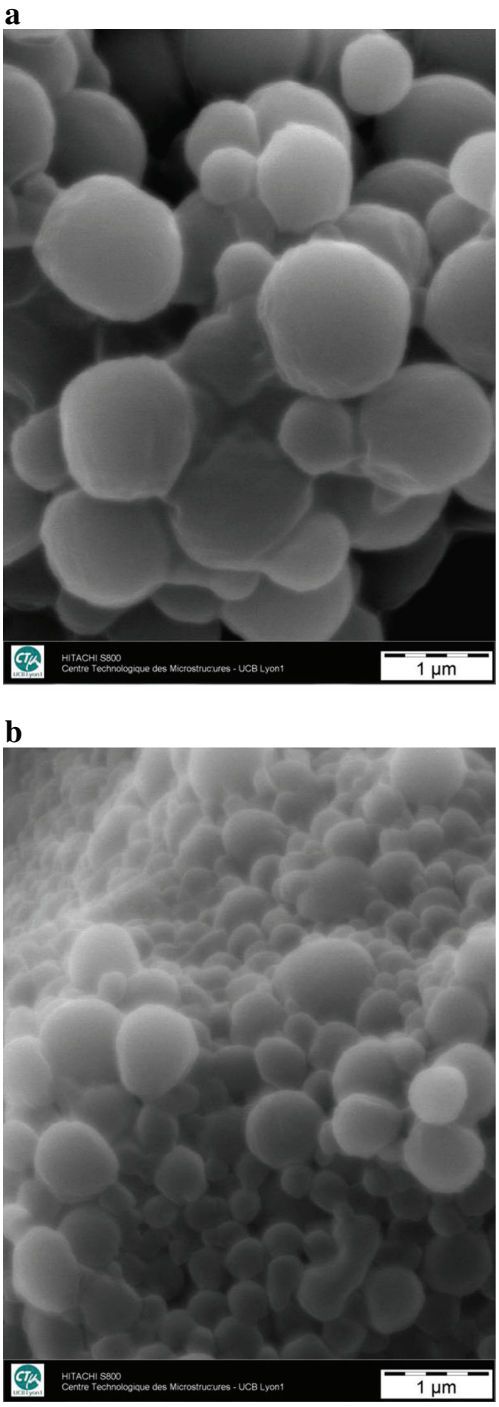

Fig. 12 **a** SEM image of nanoparticle prepared 4 min of sonication in the second step of emulsification. **b** SEM image of nanoparticle prepared 8 min of sonication in the second step of emulsification

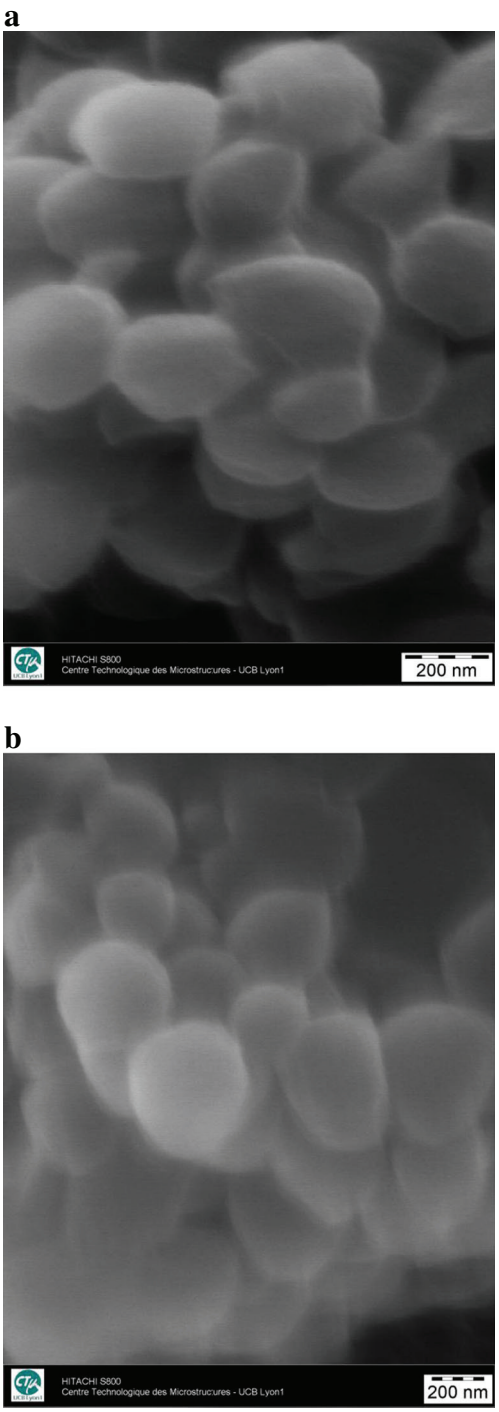

Fig. 13 **a** SEM image of nanoparticle prepared with 100-ml outer aqueous phase (W_2). **b** SEM image of nanoparticle prepared with 150-ml outer aqueous phase (W_2)

and phase volume ratio has no significant effect on zeta potential of PCL particles. The zeta potential was found in between 0 and −2 mV, which can be considered in zero range and consequently reflects noncharged particles due to noncharged nature of PCL. Similar tendency has been already reported in literature [44].

Morphology of nanoparticles

The morphology of the prepared particles was investigated via SEM. From these images, the obtained particles were found to be spherical in shape with smooth and homogeneous surfaces. SEM images also showed the slight polydisperse property of

the prepared particles as clearly evidenced in Fig. 9a. It is interesting to notice the contact among particles from the SEM images; these contacts among particles can be attributed to PVA, which has sticky nature and is difficult to remove completely [45].

The effects of modification in different preparation conditions on particle morphology are discussed below:

Effect of PCL amount on nanoparticle morphology

The amount of PCL has a very vital influence on the nanoparticle size, number, and morphology. In this study, when 1 g of PCL was used for preparation of particle dispersions, consequently more homogenous and smaller particles were observed from SEM images with comparatively narrow size distribution (Fig. 8a); on the other hand, when the PCL amount was increased to 3 g, then large size particles were observed (Fig. 8b).

Effect of ultrasound amplitude

Several samples of particles were prepared with different amplitude of ultrasound in the second step of emulsification process. From SEM images, it was observed that the recipe prepared with 60 % amplitude has several populations of particles as shown in figure (Fig. 9a) with broad size distribution. On the other hand, the particles prepared with 70 % amplitude were more uniform as illustrated (Fig. 9b) and of smaller average particle size (Table 2). This can be attributed to increase of shearing energy which induced high dispersion and fragmentation and then decreases the average particle's size, as reported by Gaikwad et al. that when the amplitude is high, smaller sizes were obtained [40].

Effect of PVA concentration

Initially, low PVA amount (i.e., 0.05 %) was used, and the SEM image shows clearly distinct particles as illustrated (Fig. 10a). While in subsequent sample, using 0.5 % PVA, the formed particles appear to be attached to each other (agglomeration) due to excessive PVA (0.5 %). The observed phenomenon is due to residual PVA which acts as plasticizer. PVA residues are on surface of the nanoparticles and are very difficult to remove completely even after washing [45] (Fig. 11).

Ultrasound exposure time (first step)

Several set of experiments were performed with different ultrasound exposure time in the first step of nanoparticle preparation by emulsification. From SEM images of nanoparticles prepared at 6-min ultrasound exposure time in the first step of emulsification (Fig. 11a) and nanoparticles prepared at 10 min (Fig. 11b), it can be concluded that both samples have almost same morphology and size. The particle size was also confirmed by light scattering analysis. The particle average size prepared at 10-min sonication was found to be 344 nm while particles prepared with 6-min sonication time was 379 nm.

Ultrasound exposure time (second step)

Similarly, effect of ultrasound exposure time in the second step of emulsification process on the particle morphology was also observed. The SEM images showed that the nanoparticles of both samples are spherical with smooth surfaces, and the sizes of particle prepared at 4-min sonication exposure are obviously larger than the particles prepared at 8-min sonication time (Fig. 12a).

Outer aqueous phase (W_2) volume fraction

The outer aqueous phase plays an important role in determination of size, morphology, and size distribution of nanoparticles. In this part, different volume fractions of outer aqueous phase were used during preparation of particles. It was shown from SEM images that, in sample with 100-ml outer aqueous phase, the particles were somewhat attached to each other and were oval in shape (Fig. 13a). On the other hand, when 150 ml of outer aqueous phase was used, spherical particles were observed (Fig. 13b). The high-degree particle attachment in sample with 100-ml aqueous phase (Fig. 13a) may be due to addition of low volume of dispersion phase (W_2) in this recipe, which may lead to high chance of collision among particles due to high viscosity, subsequently resulting in flocculation.

Conclusions

PCL nanoparticles were successfully prepared at different conditions by double emulsion solvent evaporation-like process using power ultrasound. The influence of different process parameters on the nanoparticle characteristics like morphology, zeta potential, and particle size was investigated. From this systematic study, it has been demonstrated that ultrasound emulsification is an efficient method to obtain nanoparticles via double emulsion solvent evaporation technique. Typical results are comparable with those of high-speed homogenizers like ultra-turrax. During emulsification process by power ultrasound, sonication amplitude has a significant effect on resultant nanoparticle size and morphology. Final particles have been found to be related to sonication amplitude in the second step of emulsification process. In the second step, smaller size particles were obtained at higher amplitude of ultrasound. It was reported that modification in ultrasound

exposure time has no significant impact on mean particle size in the first step of double emulsion process, while during the second step, an increased ultrasound exposure time has led to smaller size particles. In case of polymer amount, small size (235 nm) nanoparticles were obtained when 1 g of PCL was used as compared to 5 g of PCL (748 nm). It was concluded that presence of PVA (stabilizer) is compulsory for NP preparation by this process, and the particle size decreases by increasing PVA concentration from 0.05 to 0.2 %, and beyond this value, there was no significant effect on mean particle size. The relative volumes of aqueous phases are also an important factor in nanoparticle preparation process; from this study, it was established that the particle size decreases significantly with an increase of outer aqueous phase volume phase volume from 50 to 150 ml, and beyond this value, no marked effect was observed. From SEM image observation, it was found that the surface of obtained nanoparticles can be assumed to be spherical with smooth surface, having broad size distribution, but results are comparable with other pressure homogenizers. Moreover, the change in preparation condition has no effect on the zeta potential of PCL nanoparticle prepared by this process.

After this systematic studies via power ultrasound, further evaluation study has to be performed in order to determine suitable amount of active ingredient such as proteins and anticancerous molecules to be loaded in the inner aqueous phase (W_1), and also to study the release rate of active medicaments from these nanoparticles and its loading efficiency.

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III.3. Encapsulation of fluorescent nanoparticles in poly-caprolactone particles to be used as contrast agent

General summary

Bioimaging has become a powerful technique in biomedical research recently due to its unique abilities to visualize the morphological details of specific cells or tissues. Several imaging techniques have been used such as computed tomography, magnetic resonance imaging, positron emission tomography, single photon emission CT, ultrasound and optical imaging. These techniques are, generally, complementary rather than competitive. Fluorescence-based techniques have been extensively used in biological imaging due to their features of high sensitivity, selectivity, convenience, and non-invasive approach. Fluorescence microscopy depends upon the inherent property of the fluorophore. When the fluorophore is excited by light of specific wavelength (visible light spectrum), it excites electrons from the ground state to a higher energy singlet state. The excited state exists for a very short time and return to ground state by emitting light of large wavelength. This difference in wavelength is called the Stokes shift. The Stokes shift is fundamental to the sensitivity of fluorescence techniques. Most of imaging techniques depend on contrast agent to visualize the different tissues, which augment the efficiency of imaging techniques by highlighting the differences between tissues. However, most of the currently used organic fluorescent dyes (contrast agents) have some limitations, such as: (i) They cannot fluoresce continuously for long periods of time for bioimaging observations because of their rapid photobleaching (instability). (ii) Majority of organic fluorophores have a relatively broad emission spectrum i.e they can overlap with the emission spectra of other fluorophores. (iii) They reach in low concentration at target site, which consequently affect image quality. (iv) Faces problem of poor target specificity during diagnosis of diseases.

Thus, encapsulation of fluorescent contrast agents have overcome many of the limitations of conventional contrast agents (organic dyes), it can offer a better fluorescent contrast agent, with desired chemical and optical properties, such as, in vitro and in vivo stability, surface modification, high photostability, high quantum yield, large stokes shift, resistance to metabolic disintegration and non-toxicity, and flexible processability in order to be further conjugated with various biomolecules and fluorophores. Moreover encapsulation of fluorescent agent with polycaprolactone could provide protective layer of a nontoxic and biocompatible material around dye molecules, reducing the penetration of oxygen molecules thus improve it photostability. The surface of polycaprolactone polymer can be easily modified to attaché different ligands and biomolecules.

In this study, the fluorescent polymer nanoparticles (as a model) were encapsulated by the modified double emulsion ($W_1/O/W_2$) solvent evaporation technique, using two-step emulsification via sonication. A set of formulations were prepared by incorporating different concentrations of fluorescent nanoparticles (FluoSpheres®) in the inner aqueous phase (W_1). In the first step, W1 was then added to polycaprolactone solution in dichloromethane (3 g in 12 ml) and homogenized to form primary emulsion. In the second step, the primary emulsion was homogenized with 0.5% polyvinyl alcohol solution (as outer aqueous phase). Subsequently, the organic solvent evaporation from the dispersion with the help of rotary evaporator has led to the formation of solidified fluorescent-loaded polycaprolactone particles. These dispersed particles were then recovered by centrifugation at 10000 rpm for 10 minutes and washed three times with deionized water properly. The prepared particles were then characterized in term of particle size, SEM and TEM morphology, confocal microscopy and % encapsulation efficiency. It was shown, that the biodegradable polymer polycaprolactone is a useful nano- and micro carrier for imaging agents that can be used in diagnosis of various daisies. It was observed that the presence of fluorescent contrast agent in formulation has no significant effect on the colloidal properties of the final particles. Both, fluorescent-loaded particle and blank particles were analyzed for average particle size and it was found that the presence of fluorescent agent in particles did not affect the particle size when used in different concentrations. As the fluorescent nanoparticles were used in very small amount because they are highly sensitive and are effective in very minute concentration. Also, zeta potential of various formulations was determined at 25°C, using Malvern autotitrator MPT-2, and it was found to be stable at different pH values (pH 3, pH 5, pH 7, pH 9, and pH 11), which reveals the non-charged nature of the polycaprolactone. The particle morphology was observed under TEM, and the fluorescent-loaded submicron particle was seen to be spherical and fairly detached from each other and no impurities were observed in TEM images. The SEM images showed that the nanoparticles produced were in submicron size (< 400 nm) and had spherical shape with smooth texture. The relatively smooth surface of the particle supported the assumption that the release of the encapsulated moiety may be caused by matrix erosion. The incorporation of fluorescent nanoparticles in different concentrations did not affect the morphology of submicron particles. The percentage loading efficiencies of the contrast agent were found in-between 84.4 % to 91.4 % in various formulations. The encapsulation efficiency was almost same for all the formulations containing various concentrations of contrast. Also, with

an increase in concentration of contrast agent, there was insignificant increase in EE from 84.4% to 91.4%. Furthermore, CLSM images showed that all particles are labeled and contrast agents are dispersed in polymer matrix. Although several fluorescent contrast agents have been encapsulated and applied biologically, still further research should be done before they can be widely employed as fluorescent probes in clinical trials. With further progresses in design and synthesis of high class multifunctional fluorescent particles, their extensive application may be expected in theranostics, which could include encapsulation of hydrophilic/hydrophobic or both drugs along with one or more fluorescent nanoparticles simultaneously. And, with the help of imaging technique, tracking of loaded drug, and drug distribution in target tissues would be possible.

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Submicron polycaprolactone particles as a carrier for imaging contrast agent for in vitro applications

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ABSTRACT

Fluorescent materials have recently attracted considerable attention due to their unique properties and high performance as imaging agent in biomedical fields. Different imaging agents have been encapsulated in order to restrict its delivery to a specific area. In this study, a fluorescent contrast agent was encapsulated for in vitro application by polycaprolactone (PCL) polymer. The encapsulation was performed using modified double emulsion solvent evaporation technique with sonication. Fluorescent nanoparticles (20 nm) were incorporated in the inner aqueous phase of double emulsion. A number of samples were fabricated using different concentrations of fluorescent contrast agent. The contrast agent-containing submicron particle was characterized by a zetasizer for average particle size, SEM and TEM for morphology observations and fluorescence spectrophotometer for encapsulation efficiency. Moreover, contrast agent distribution in the PCL matrix was determined by confocal microscopy. The incorporation of contrast agent in different concentrations did not affect the physicochemical properties of PCL particles and the average size of encapsulated particles was found to be in the submicron range.

1. Introduction

Recently, biomedical imaging has received immense attention due to its extensive applications in diagnosis of disease at an early stage [1,2], tracking of therapeutic carrier, monitoring disease changes and determining a proper end state to therapy [3]. In many cases, imaging is performed for diagnosis of a disease state prior to initiation of therapy. Several imaging techniques such as, computed X-ray tomography (CT), optical imaging, magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon-emission computed tomography (SPECT), and ultrasound are being used for diagnosis of disease including cancer and neurodegenerative diseases. These are noninvasive techniques and allow the visualization of target tissues [4,5]. Various imaging technologies (Magnetic resonance, optical etc) depend on contrast agent to visualize the organ of interest [6]. Contrast agents could augment the efficiency of imaging techniques by highlighting the differences between tissues [3], without contrast agent such information-rich images would be unobtainable. The contrast agents currently used for diagnosis faces problems of poor target specificity, instability and low concentration at target site, which consequently affect image quality. Thus, it is essential to deliver high payload of contrast agent specifically to an organ of interest in order to obtain beneficial images. Due to their specific size and shape, submicron particles offer multifunctional capability. Polymeric particles, incorporated with contrast agents (polymeric encapsulation of contrast agent) are emerging as a new class of imaging agent for detecting human diseases [7]. These particles have shown many potential benefits, such as, (i) they restrict the delivery of imaging agent to a small area thus reducing the systemic side effects (ii) they can deliver high payload of imaging agent at target site selectively (iii) they can travel through blood vessels and protect the encapsulated agent until delivery (iv) polymeric particles provide high surface area that allows the attachment of appropriate targeting agent and enhance the release properties (v) they can modify the biodistribution of active agent in controlled manner. Moreover, polymeric materials have the ability to encapsulate different contrast agents and active molecules in a single particle enabling multifunctional particles possibilities [6–9], with a capacity for targeted site imaging and delivery of therapeutic agents [8]. Standard process allow

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for the encapsulation of lipophilic molecules into a multitude of particulate materials, however their application to hydrophilic compounds encapsulation is limited due to uncontrolled leakage of entrapped compounds during the preparation process [10–12]. However, double emulsion technique is an appropriate method for the encapsulation of hydrophilic molecules as well as hydrophobic molecules, additionally, it allows flexibility in particle size by adjusting process parameters, the process is independent of special laboratory equipment, the operation costs are low and preferable for low scale production [13,14]. Several polymeric materials such as polystyrene, dextran, chitosan poly (lactic acid) and poly (lactic-co-glycolic acid) has been used to develop multi-target and multifunctional particle loaded with fluorescent agent for optical imaging [15]. The characteristics (size, surface charges, and structures) of these particles can be controlled by polymeric backbone and process parameter during preparation, in order to improve the fluorescent agent entrapment, blood circulation time, target site accumulation and target specificity of imaging particles probe [16,17]. Polycaprolatone is a biodegradable polymer with low glass transition temperature and melting point and the polymer metabolites are eliminated from the body by innate metabolic process [18]. Due to biodegradable, biocompatible and non-toxic nature of PCL, it is extensively studied for control drug delivery system in several formulations including nanoparticles, implants, nano-fibers, microspheres etc. Its compatibility with wide range of drug and its slow degradation to release drug for extended period of time (months–years) makes it an appropriate candidate for controlled drug delivery systems. Moreover, PCL versatility is due to the fact that, it allows the modification of its physicochemical and mechanical properties by copolymerization, which intern affect all other properties of PCL such as solubility, ionic property and degradation pattern [19–21]. Though, PCL has been extensive investigate in drug delivery system [22–24], but its applications in imaging technologies are studied too little, especially for encapsulation of contrast agent in optical imaging techniques.

Confocal laser scanning microscopy (CLSM) can be used as potential tool for characterization of polymeric particles. It allows visualization of structures not only on surface, but also inside the particles without prior sample destruction and can be used to visualize the encapsulated compounds. CLSM has ability to acquire in-focus images from selected depths, allowing three-dimensional reconstructions of topologically complex objects, by assembling several coplanar cross sections and already has been used for evaluation of different formulations [25,26]. Conversely, SEM does not allow the visualization of internal structures (encapsulated phase) of intact particle, and requires mechanical section of particle to observe the internal structures, which may result in loss of encapsulated phase. Moreover, CLSM enable us to evaluate the encapsulation of fluorescent contrast agent into submicron particles as well as its distribution in biological samples. dual fluorescence technique enable us to record images at two individual wavelength couplets (excitation/emission), subsequently the confocal images of fluorescent particles (visualized under laser scanning) can be superimposed on images of submicron carrier particles (under normal observation) using the same sample plane [27]. Hence, the main goal of this work was to develop a polymeric submicron carrier for fluorescent contrast agent that might enhance stability, augment the imaging efficiency, and restrict contrast agent accumulation to specific area and could be used for delivery of imaging agent and therapeutic agent simultaneously. We studied the encapsulation of contrast agents in/on to PCL particles using double emulsion evaporation technique. Several formulations with different concentrations of contrast agent were characterized regarding particles size, morphology, zeta potential, encapsulation efficiency etc. Average size of particles was found to be in submicron range with smooth surfaces, spherical shapes and high encapsulation efficiency. We also evaluated the penetration of the particles into excised human skin.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL) (Mw = 14000 g/mol), polyvinyl alcohol (PVA) (Mowiol® 4–88, Mw = 31000 g/mol), and dichloromethane (DCM) were obtained from Sigma–Aldrich, Germany and used as such. Water was deionized using (Aquadem® from Veolia Water, France). Ultrasonic homogenizer system "CY-500" ivymen® (500W, 20 kHz) from SELECTA GROUP, Switzerland. Analytical balance (Acculab ALC-110.4) was supplied by Sartorius group, Germany. Hitachi S-800 FEG Scanning Electron Microscope from Hitachi Japan, Zetasizer Nano-ZS (Malvern, UK), red fluorescent (580/605) labeled carboxyl-functionalized polystyrene particles (FluoSpheres®) was purchased from Molecular Probes® F-8786 (Oregon, USA). CM 120 Transmission electron microscope was obtained from Philips, Netherlands. Eppendorf 5415C Centrifuge, was obtained from Eppendorf, Germany, and Rotary Evaporator (1500 W) was supplied by Nahita. Cary Eclipse Fluorescence Spectrophotometer (Fluorometer) was obtained from Agilent Technologies (Malaysia).

2.2. Methods

2.2.1. Preparation of submicron particles incorporated with contrast agent

The fluorescent contrast agent was encapsulated by the modified double emulsion ($W_1/O/W_2$) solvent evaporation process, via two-step emulsification technique using power ultrasound as described by Iqbal et al. [28]. Briefly, before preparing the first emulsion, the inner aqueous phase (W_1), was prepared by incorporating different concentrations of fluorescent contrast agent (FluoSpheres®) in deionized water and the volume was made up to 1.5 ml. Similarly, oil phase was prepared by dissolving 3 g of polycaprolactone (PCL) in 12 ml of dichloromethane properly to form a clear solution. And PVA solution (0.5%) was prepared to be used as outer aqueous phase (W_2), by taking 5 g of PVA in 1000 ml flask and sufficient amount of deionized water was added to make up the volume. PVA was dissolved under magnetic stirring at 60 °C for 40 min, which resulted in a clear PVA solution.

Then, in the first step of emulsification, the inner aqueous phase (W_1) was added to PCL solution and this mixture was homogenized properly to form a primary emulsion (W_1/O) using ultrasonic homogenizer "CY-500" ivymen® at a 70% amplitude for 5 min. In the second step, the primary emulsion (W_1/O) was dispersed in 150 ml of the outer aqueous phase (W_2) containing 0.5% PVA as stabilizer in a 250 ml glass beaker. This mixture was homogenized by an ultrasonic probe at 70% amplitude for 8 min, to produce a double emulsion ($W_1/O/W_2$). The ultrasonic horn was positioned 2 mm above the oil-water interface in the system. This position was kept constant for all the experiments. Afterward, the organic solvent evaporation from the dispersion with the help of rotary evaporator has led to the formation of solidified PCL particles. These dispersed particles were then recovered by centrifugation at 10000 rpm for 10 min and washed three times with deionized water properly. The ultrasonic transducer (homogenizer) consisting of titanium alloy probe (5.6 mm diameter and 60 mm height) used has power of 500W and frequency of 20 kHz. The above mentioned conditions were same for all the experiments, only the concentration of fluorescent agent was changed in each formulation.

2.3. Physicochemical characterization of submicron particle

2.3.1. Hydrodynamic size measurement

The hydrodynamic particles size (D_h) of the colloidal dispersions was determined by dynamic light scattering using zetasizer from Malvern Instrument at room temperature (25 °C) and in 10^{-3} M NaCl concentration. The mean hydrodynamic diameter is calculated by using the Stokes–Einstein's equation:

$$D_h = \frac{kT}{3\pi\eta D} \quad (1)$$

where, k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium, and D is the diffusion coefficient. Each sample was prepared by adding one drop of submicron particles dispersion in about 1.5 ml of deionized water in quartz cell and then the cell was placed in zetasizer for analysis. Mean particle size was determined at a scattering angle of 90° using appropriately diluted samples. For each preparation, the mean size of three determinations was calculated.

2.3.2. Zeta potential

Prepared particles were also characterized with respect to electronic mobility and zeta potential using a zetasizer (Nano-ZS, Malvern). Analysis was performed at different pH values (pH 3, pH 5, pH 7, pH 9 and pH 11) at 25 °C, using Malvern autotitrator MPT-2. All samples were appropriately diluted with 1 mM NaCl aqueous dispersant in order to maintain constant ionic strength. For each sample the mean value of three determinations were established. Electrophoretic mobility is converted into zeta potential by using Smoluchowski's equation:

$$\mu_e = \frac{\epsilon}{4\pi\eta}\zeta \quad (2)$$

where, ϵ is the dielectric constant, η is the viscosity of the medium, and ζ is the zeta potential.

2.3.3. Determination of encapsulation efficiency

The encapsulation efficiency refers to the amount of fluorescent contrast agent encapsulated into the PCL polymeric particles as compared to the total amount of fluorescent material added in formulations. For determination of encapsulation efficiency, indirect method was used i.e by measuring the amount of fluorescent agent that was not entrapped and, thus, remained in the supernatant layer upon centrifugation of particulate dispersion. The encapsulation efficiency of fluorescent contrast agent in PCL particles was then calculated in triplicate using indirectly method, as follow:

$$\text{Encapsulation effeciency}(\%) = \frac{\text{Totat amount FC added} - \text{Amount of recovered in supernatents}}{\text{Total amount of Fluorescent agent added}} \times 100 \quad (3)$$

where, *FC* is the fluorescent contrast agent. For this purpose, specific amount of particulate dispersion were centrifuged (Eppendorf 5415C; Eppendorf, Germany) at 14000 for 10 min, and the supernatant was then analyzed for fluorescent intensity by adding about 2.5 ml sample into fluorometer's covet. The excitation and emission wavelengths used were 580 nm and 605 nm respectively. All the samples were measured at PMT detector voltage of 600 V, with emission and excitation slits width of 5 nm for each. Before encapsulation efficiency measurement of prepared particles, a standard curve was generated by preparing a series of fluorescent agent dilutions in deionized water, these dilutions were analyzed for fluorescence intensity via fluorometer and, the results obtained were used to produce the standard curve. Afterward, the amount of fluorescent contrast agent (percent encapsulation efficiency) in different formulations was quantified by using this standard curve. When the fluorescence spectra of contrast agent dispersed in water was analyzed by fluorometer, their excitation and emission maxima were obtained at 580 nm and 606 nm respectively, which is almost the same as mentioned on the label of FluoSpheres® (λ_{ex} = 580, λ_{em} = 605).

2.3.4. Particles morphology

Scanning Electron Microscopy (SEM) and transmission electron microscopy (TEM) were performed in order to determine the shape and surface morphology of the fluorescent-containing polymeric particles. Scanning Electron Microscopy (SEM) morphological evaluation was performed with Hitachi S800 FEG microscope at the "Centre Technologique des Microstructures" (CTμ) at the University of Lyon (Villeurbanne, France). A drop of diluted aqueous suspension of submicron particles was deposited on a flat steel holder and dried at room temperature. The sample was finally coated under vacuum by cathodic sputtering with platinum (5 nm). The samples were observed by SEM under an accelerating voltage of 15 kV. Before deposition on steel holder, all samples of particle were centrifuged at 1000 rpm for 10 min and washed three times with deionized water. While, the transmission electron microscopy (TEM) of the fluorescent agent encapsulated particles were done by a Philips CM 120 Transmission electron microscope (CMEABG, Claude Bernard University Lyon 1, France) at an electron accelerating voltage of 100 kV. A drop of highly diluted sample was deposited onto a copper grid covered with a 200 mesh and covered with formvar carbon membrane and dried at room temperature before TEM analysis.

2.3.5. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is an optical imaging technique that can be used to achieve cellular resolution in real-time and record depth section information of tissue with cellular definition. CLSM allows the inspection of internal structures of fluorescent-containing polymeric particles without prior sample destruction and it can be used for localization of encapsulated compounds [26,29]. Fluorescence technique enable us to record images at two individual wavelength couplets, subsequently the confocal images of fluorescent nanoparticles (visualized at a specific wavelength) can be overlaid on images of carrier particles (visualized at white light) obtained in the same sample plane [27]. An ideal fluorescent agent selected for CLSM might possess good quantum efficiency, high selectivity for target site, high resistance to photobleaching, least disturbance to the sample and minimum cross-talk when many contrast agents are used together. When a fluorescent agent is excited by light of specific wavelength, it remains in excited state for only a few nanoseconds and then relaxes into its ground state by emitting fluorescence of longer wavelength. The intensity of energy emitted by fluorescent agent at its optimum excitation wavelength can be described by quantum efficiency value (QE) value of the fluorescent agent [30,31].

$$QE = \frac{\text{Energy emitted}}{\text{Energy absorbed}} \quad (4)$$

Several samples were prepared by incorporating different concentrations of fluorescent contrast agent in the inner aqueous phase (W_1) of double emulsion. Additionally, one sample was prepared without adding contrast agent (blank sample). After preparation of all formulations, the particulate dispersion was visualized by confocal laser scanning microscopy (CLSM).

Confocal microscopy was performed at the "Centre Technologique des Microstructures" (CTμ) at the University of Lyon

(Villeurbanne, France), on the Axiovert 200 LSM 510 Meta microscope (Carl Zeiss, Jena, Germany) using a 63x oil immersion objective of 1.4 Numeric Aperture (N.A.). The fluorescence emission was collected with a pinhole at 136 μm, the 543 nm laser was set up at 70% of its maximum intensity and the emission was collected from 560 nm and above. The contrast agent (FluoSpheres®) used was red fluorescent beads/nanoparticles, maximally excited at 580 nm and their emission wavelength was 605 nm. These particles were supplied as suspensions (2% solids content) in water, with average diameter of 20 nm.

2.4. Skin penetration of particles

Fresh human skins from surgery of healthy Caucasians were used. The surface was cleaned with water and 100 μl of PCL particles were deposited. After application for 48 h, discs of skin, 3 mm in diameter, were punched out, frozen and embedded in Tissue-Tek® O.C.T. Cryostat sections (10 μm) perpendicular to the skin surface were prepared and mounted on poly-lysine coated slides. Tissue sections were examined by CLSM.

3. Results and discussion

The physicochemical aspect of colloidal system such as, particle size and zeta potential, are known to influence the physical stability of colloids, release rate and their interaction with cells and biological environment [32]. The particles were characterized on the basis of morphology, particle size, zeta potential, contrast agent encapsulation efficiency and their localization.

3.1. Particles size

The hydrodynamic particle size of each sample was examined. Each sample was highly diluted before any analysis. The presented mean particle size was at least the average of 3 independent measurements. The size range of particles produced by $W_1/O/W_2$ protocol can be adjusted depending on the amplitude of sonotrode and time duration of sonication [33]. The prepared particles were analyzed via zetasizer and, their average size was found to be in submicron range (Table 1). The presence of fluorescent agent in PCL particles did not affect the particle size when used in small concentrations (F2, F3 and F4), while, at relatively higher concentration (F6 and F7) there was a slight increase in particle size but it was insignificant (Table 1). This can be attributed to the constant volume of inner aqueous phase used in the all formulations. And the results showed that the chemical composition of the inner aqueous phase has no significant effects on the final particle size.

3.2. Zeta potential (ZP)

The zeta potential reflects the strength of the colloidal particles electrical barrier and is used as a vital parameter in evaluating the stability of colloidal dispersions [34]. In order to point out the effect of pH on the particles surface charge density, the zeta potential of PCL particles was determined as a function of pH, with different concentrations of contrast agent-containing samples and a blank sample. It was measured at different pH values, and from the results obtained it has been shown that, the zeta potential of contrast agent loaded particles varied between 0.1 and −7.4, which could be considered near to zero. These results demonstrated that there was no considerable variation in zeta potential of different formulations as the pH was increased (Fig. 1), which can be attributed to uncharged chemical nature of PCL as already reported in literature [35,36] and also to the screening effect of PVA. Normally, for charged particles, the use of non-charged stabilizer leads to the surface charge

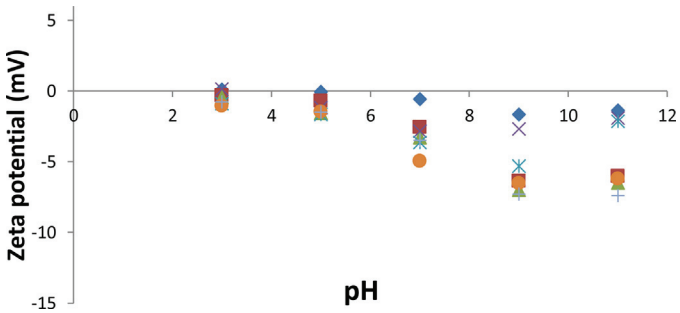

Fig. 1. Zeta potential versus pH of Contrast agent-containing (CA) submicron particles.
(◆) CA free particles, (■) CA 0.06 μg/g of PCL, (▲) CA 0.16 μg/g of PCL, (×) CA 0.33 μg/g of PCL, (✳) CA 0.50 μg/g of PCL, (●) CA 0.66 μg/g of PCL, (+) CA 1.33 μg/g of PCL.

screening effect and shift in sleeping plan position far from particles surface, which induce decreases in the absolute of the zeta potential.

3.3. Morphology

Scanning electronic microscopy (SEM) was used to visualize the morphology of contrast agent-loaded PCL particles prepared by $W_1/O/W_2$ double emulsion evaporation technique at 70% sonication amplitude. Particles were evaluated on the basis of shape, surface texture, smoothness and presence of inter-particulate bridging. Under SEM observations, the submicron particles produced had smooth surfaces and spherical shapes (Fig. 2) with an average particle size in submicron range. The average particle sizes were also confirmed by light scattering analysis (Table. 1). The relatively smooth surface of the particle supported the assumption that the release of the encapsulated moiety may be caused by matrix erosion [37]. Moreover, rarely a split or broken particles were seen within all samples. The incorporation of contrast agent in different concentrations did not affect the morphology of submicron particles. SEM Images showed slight bridging between some particles, this can be attributed to sticky nature of residual stabilizer (PVA) used in the formulations; since, it is difficult to remove PVA absolutely even after washing [38].

Additionally, TEM was used to observe the produced particles formed by double emulsion method. The loading of fluorescent contrast agent in various concentrations into the PCL particle did not affect the morphology of the particles as shown in Fig. 3. Under TEM observations, no inorganic impurities were found in the samples and, the submicron particle appeared to be spherical and fairly detached from each other, as the samples were highly diluted prior to TEM observation.

3.4. Encapsulation efficiency (EE)

Encapsulation of fluorescent contrast agents can be of wide interest in optical imaging, because it decreases photobleaching, prevent dye aggregation and increases fluorescence per particle by confining large no of fluorescent molecule into a small volume [39]. Though once the fluorescent material is incorporated into polymeric particles, then dispersion medium is not in direct contact with fluorescent agent, therefore, medium of dispersion does not affect the fluorescence spectra [40]. The encapsulation efficiency of fluorescent contrast agent into PCL submicron particles was determined by indirect method using cary eclipse fluorescence spectrophotometer. All the measurements were performed in triplicate.

Table 1
Composition of various formulations for preparation of submicron particle incorporated with fluorescent contrast agent, particles were prepared via two-step double emulsion evaporation process. Double emulsion was homogenized by sonication at 70% amplitude for 5 min in the first step, and 8 min in the second step of emulsification. Samples were prepared by incorporating different concentrations of contrast agent (FluoSpheres®) along with one blank sample (F1) i.e without contrast agent. The average particle size and encapsulation efficiency (%) of these formulations are also tabulated.

Run	FluoSpheres®(μg/g of PCL)	Inner aqueous phase (ml)	PCL polymer (g)	PVA solution (0.5%, ml)	Particle size (nm)	Encapsulation efficiency (%)
F 1	0	1.5	3	150	342	NA[a]
F 2	0.06	1.5	3	150	346	84.4
F 3	0.16	1.5	3	150	354	86
F 4	0.33	1.5	3	150	347	89.8
F 5	0.50	1.5	3	150	322	88.6
F 6	0.66	1.5	3	150	371	90.2
F 7	1.33	1.5	3	150	375	91.4

[a] Not applicable.

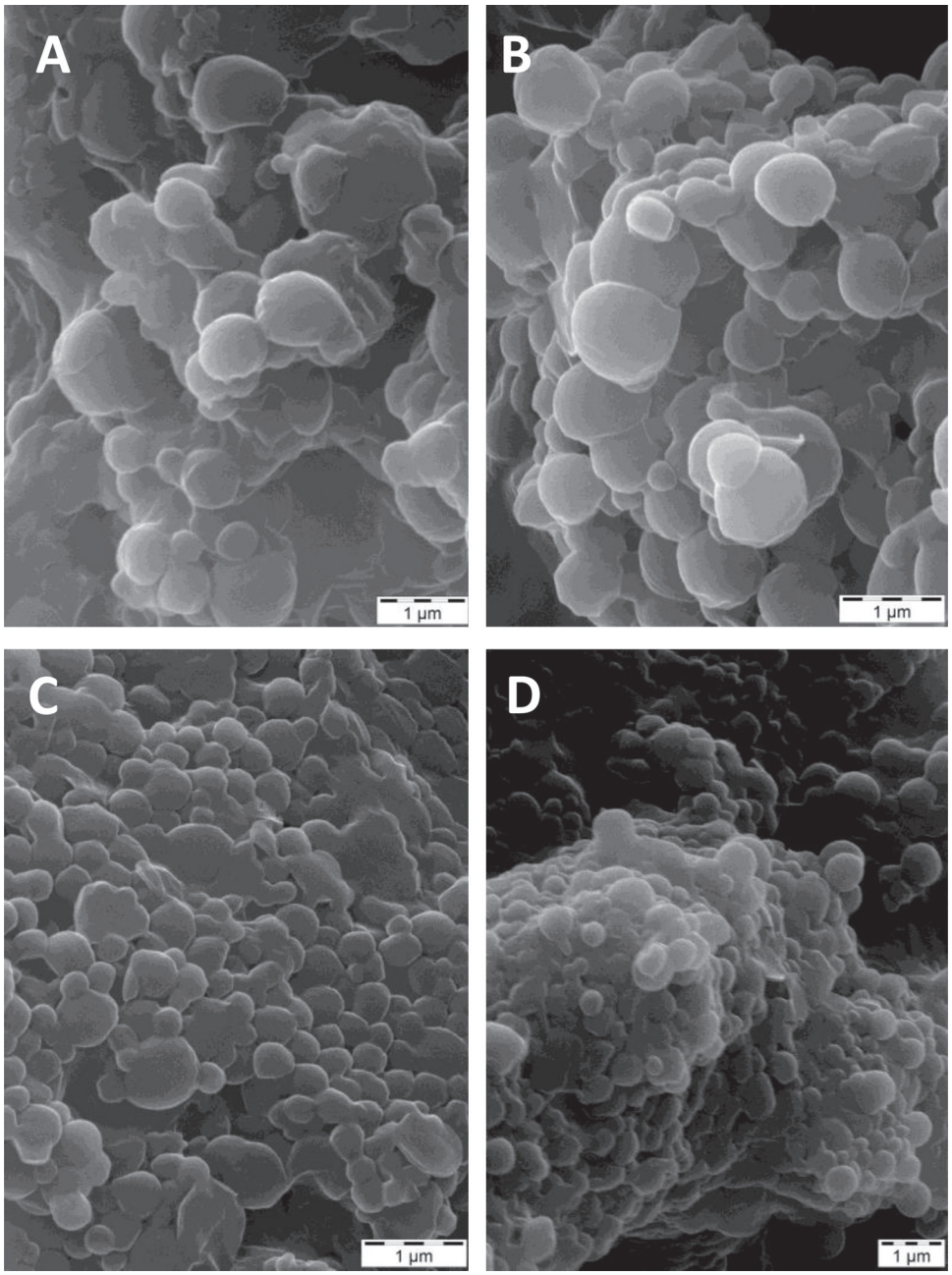

Fig. 2. SEM images of PCL particles incorporated with different concentrations of contrast agent. (A) 0.06 μg/g of PCL (B) 0.66 μg/g of PCL (C) 1.33 μg/g of PCL (D) blank formulation. The scale bars represent 1 μm.

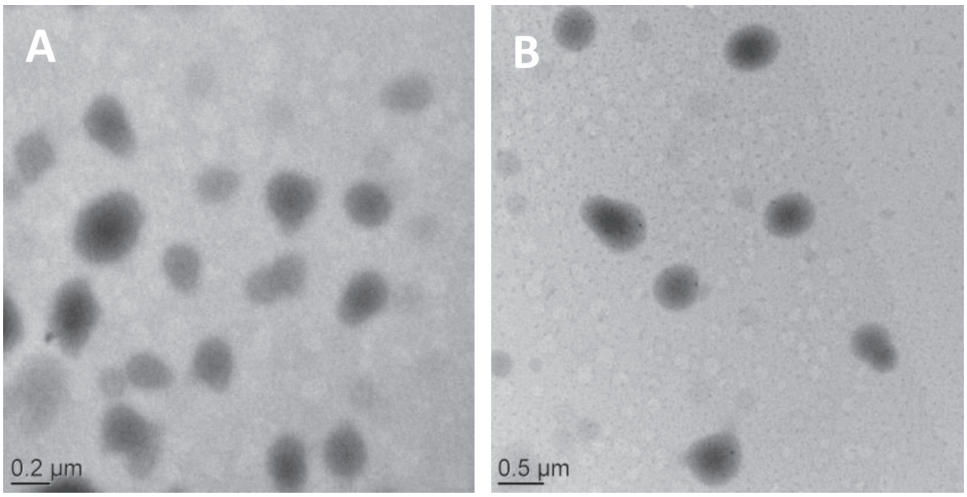

Fig. 3. TEM representative images of contrast agent-containing PCL particles. Contrast agent was incorporated in different concentrations to various samples. (a) 0.16 μg/g of PCL and (b) 0.66 μg/g of PCL. Scale bar in A represents 0.2 μm, while in C it represents 0.5 μm.

The average encapsulation efficiencies of the contrast agent were found between 84.4% and 91.4% in various formulations (Table. 1). The percentage encapsulation efficiency of formulation F2 was found to be 84.4%, which slightly increased to 86%, 89.9%, 88.6%, 90.2% and 91.4% in the other formulations i.e. F3, F4, F5, F6 and F7 respectively (Table. 1). These results showed that encapsulation efficiency were almost same for various concentrations of contrast agent incorporated in different formulations. Also, with an increase in concentration of contrast agent, there was slight increase in EE from 84.4% to 91.4% but it was insignificant. The optical spectra of fluorescent agents are often affected by the change in polarity of their medium, which cause change in dipole moment of fluorescent agent and subsequently shift of peak maxima [41]. The background intensity was also measured; which, was found to be 11 arbitrary units (a.u.) for blank sample, and excluded from final values. The high encapsulation efficiency may be due to high affinity of hydrophilic contrast agent (CA) toward inner aqueous phase (W_1). The CA is easily disposable in W_1, and its homogenization with PCL solution (in DCM) results in appropriate nanodroplets formation during the first step of emulsification. In the second step of emulsification, the PCL in DCM solvent rapidly solidifies and encapsulating the CA in the inner aqueous phase and thus, preventing the leakage of CA from W_1 to outer aqueous phase (W_2). The PCL precipitation in the second step is induced by the diffusion of organic solvent from dispersed nanodroplets to outer aqueous phase (W_2).

3.5. Confocal laser scanning microscopy

After the encapsulation efficiency of contrast agent analysis by fluorescence microscopy, the localization and distribution of contrast agent was observed with respect to PCL particles in the system (background) with the help of CLSM. Moreover, the influence of contrast agent concentration on the fluorescence images (brightness) in different samples was also visualized. The confocal microscopy can be used either in fluorescence mode, which collect the light generated by a fluorescence contrast agent or in reflectance mode (to observe structural and morphological information of the background under white light) [42].

All samples were visualized in fluorescence mode (Fig. 4) and direct observation; afterward, these images were overlaid with the help of Zeiss LSM confocal software. The contrast agent-loaded particles of size 300-400 nm were easily visible under CLSM when excited at a proper wavelength (nm) of light. Fig. 4 shows the distribution of contrast agent (λ_{ex} = 580, λ_{em} = 605) encapsulated by PCL particles. From the overlaid images for the same sample (Fig. 4B3, C3, D3), one can see that the fluorescent contrast agents are only localized at PCL particles positions, which indicate that all particles are labeled, showing proper encapsulation of contrast agent by polycaprolactone-based particles. Moreover, from CLSM images, the fluorescence can be observed quantitatively on the basis of images brightness. For example, image 4c is brighter (due to high fluorescence) than image 4b, which confirmed the high concentration of fluorescent contrast agent incorporated in 4c formulation (0.66 μg/g of PCL) as compared to 4b (0.06 μg/g PCL) (Fig. 4). On the other hand the lack of fluorescence emission in image 4a (blank formulation) verified the absence of contrast agent in this formulation (blank sample).

3.6. Skin penetration of formulated particles

Several studies using in vitro and ex vivo models have demonstrated that nanoparticles have the potential to penetrate across the skin barrier or the follicular structure [27,43].

Fig. 5 shows the penetration pattern of particles in the skin and in the hair follicle. The red fluorescence allowed easy identification of particles. PLC particles remained on the surface of both *stratum corneum* (Fig. 5A) and hair follicle (Fig. 5B). Therefore, under passive diffusion conditions, no penetration of particles was found.

The main barrier to cutaneous molecule absorption is the impermeability of the *stratum corneum*. Another important issue is the mechanical stress applied to the skin when investigating the penetration behavior. In future works, we will evaluate the impact of the removal of the *stratum corneum* and the application of a mechanical stimulation.

4. Conclusion

The biodegradable polymer PCL is an extremely useful nano- and micro carrier for several imaging contrast agents that can be used for theranostic purpose and targeting of diseased tissue. Here, PCL submicron particles loaded with imaging contrast agent were successfully prepared via double emulsion technique in order to restrict its delivery to a specific area, prevent contrast agent aggregation and improve its stability, which could enhance the efficiency of imaging techniques. It was observed that the presence of fluorescent agent in formulation has no significant influence on the colloidal properties of the final particles.

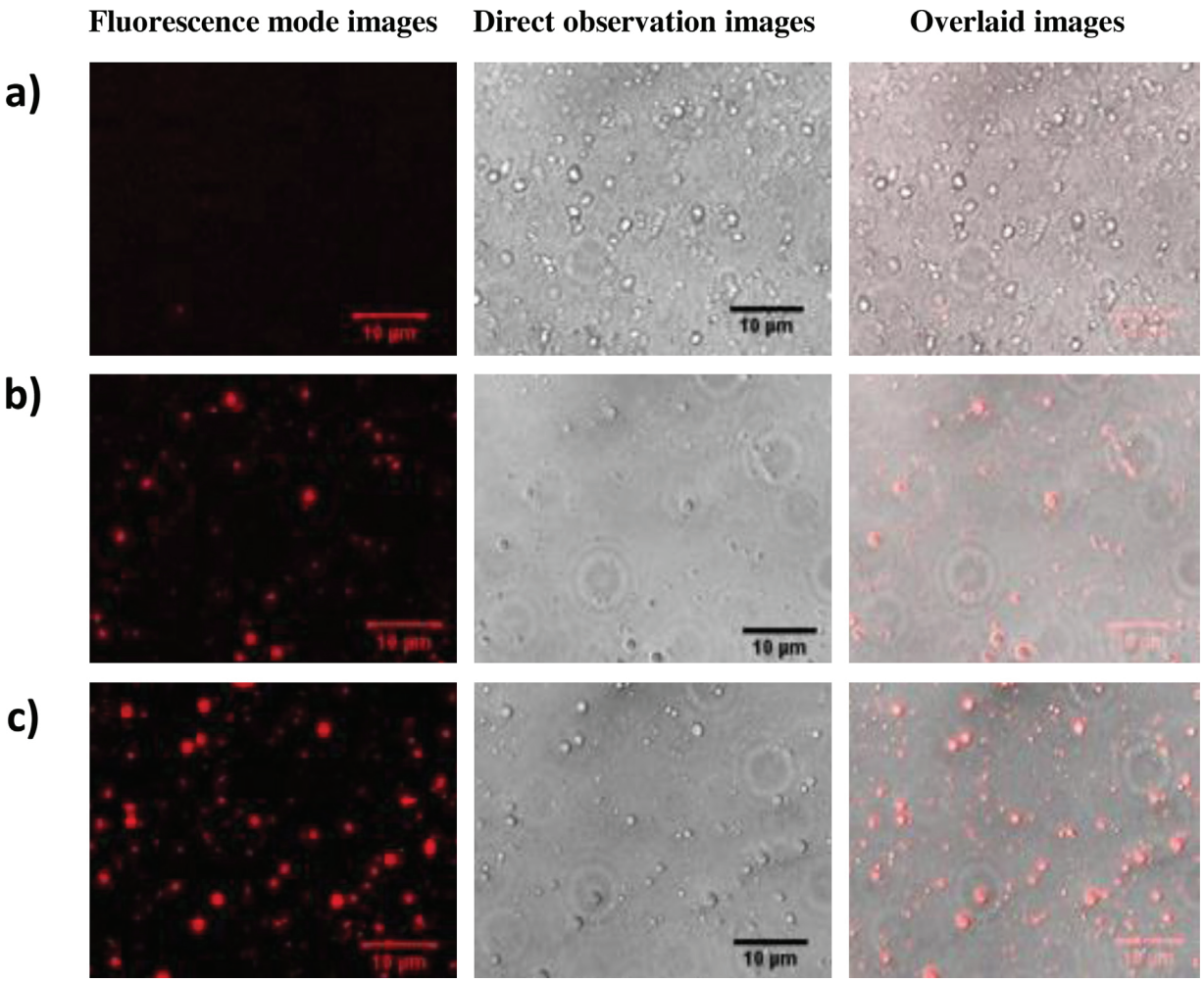

Fig. 4. Representative CLSM images of PCL particles containing different concentrations of fluorescent contrast agent (a) blank formulation (b) 0.06 μg/g PCL (c) 0.66 μg/g PCL prepared by double emulsion process. All images were taken with 63x oil immersion objective of 1.4 numeric aperture in fluorescence mode (left panel) along with their corresponding direct observation images (middle panel), afterward, these images were overlaid (right panel). The scale bars represent 10 μm.

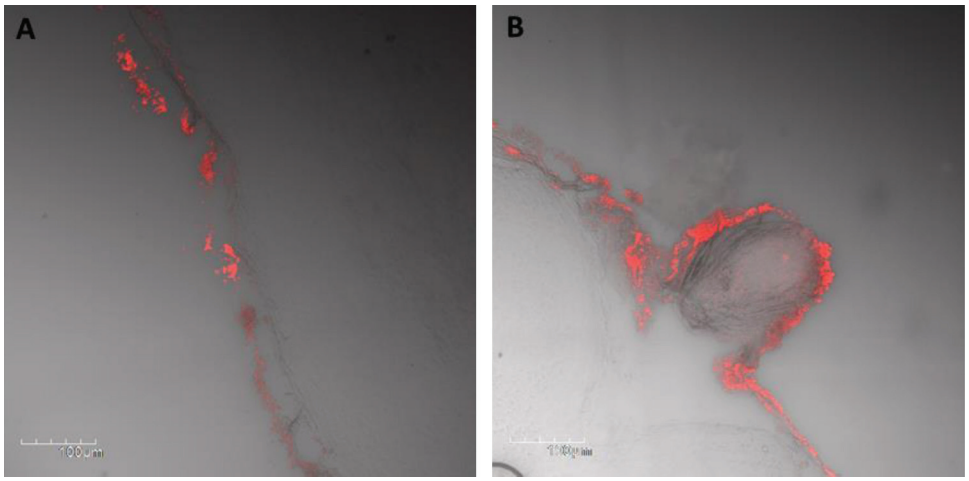

Fig. 5. Representative CLSM images of skin treated for 48 h with PCL particles. Images were taken with 20x objective in fluorescence mode along with their corresponding direct observation images, afterward, these images were overlaid (A: skin; B: follicular region). The scale bars represent 100 μm.

The prepared particles were analyzed for average particle size and it was found that the presence of fluorescent agent in particles did not affect the particle size when used in different concentrations. Also, zeta potential of various formulations was stable at different pH values.

Under TEM observation the submicron particle appeared to be spherical and fairly detached from each other and no

impurities were observed in TEM images. The SEM images revealed that nanoparticles produced were in submicron size (<400 nm) with spherical shape and smooth surface. The relatively smooth surface of the particle supported the assumption that the release of the encapsulated moiety may be caused by matrix erosion. The incorporation of contrast agent in different concentrations did not affect the morphology of submicron particles. The average encapsulation efficiencies of the contrast agent were found in-between 84.4% to 91.4% in various formulations. The encapsulation efficiency was almost same for all the formulations containing various concentrations of contrast. Also, with an increase in concentration of contrast agent, there was insignificant increase in EE from 84.4% to 91.4%. Furthermore, CLSM images showed that all particles are labeled and contrast agents are dispersed in polymer matrix.

Though, there has been significant research progress in the field of synthesis polymeric particle and their imaging application, but still faces some challenges to be focused like active targeting, burst release, toxicity, stability of contrast agent and solubility of polymeric material. As demonstrated here contrast agent-containing imaging particles can be prepared by double emulsion solvent evaporation technique. Furthermore, future application could include encapsulation of hydrophilic/hydrophobic or both drugs along with one or more fluorescent contrast agent can be incorporated in these particles simultaneously i.e for theranostic purpose. And, with the help of imaging technique, tracking of loaded drug, and drug distribution in target tissues would be possible. Moreover, there could be possibility to add molecular recognition capability to this particulate system by adding a targeting agent to the particle scheme. This technique for encapsulation of contrast agent can be extended to other imaging agents, hydrophilic drugs and hydrophobic drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2015.09.045.

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III.4. Preparation and characterization of gold nanoparticles

General summary

Preparation of gold nanoparticle and evaluation of its colloidal properties is presently a very dynamic area of research. New techniques are cautiously evolving that provide more control over the particle size, size distribution and shape of nanoparticles which make these particles very attractive for various applications. Gold nanoparticles have been investigated for many years because of their extensive use in various applications such as catalysis, photonics, electronics, optoelectronics, diagnostic, delivery, chemical, biological and biomedical sensing, surface plasmon resonance and surface-enhanced raman scattering (SERS) detection. The characteristic red color of the gold nanoparticle is due to the collective oscillation of the electrons in the conduction band, called surface plasmon resonance, which mainly depend on the shape, size and aggregation of the nanoparticle and dielectric constant of the surrounding medium. Classically, gold nanoparticles exhibit a single absorption peak in the visible range between 510 nm and 550 nm and with an increase in particle size, the absorption peak shifts to a longer wavelength. The width of the absorption spectra usually depends on the size distribution of the nanoparticles. The properties such as photostability, nontoxicity, surface plasmon resonance (SPR), easy surface functionalization, and their biocompatibility make these probes highly advantageous for biological imaging, cancer therapy, and drug delivery, immunoassay, protein assay, and detection of cancer cells.

There are two commonly used approaches for preparation of metallic nanoparticles, namely, “the bottom up approach”, which involves the association of atoms to fabricate nanoparticles, and “the top down method” which involves the constant division of bulk metals into nanoparticles. Currently, various techniques have been used for the preparation of gold nanoparticles such as, chemical, electrochemical, irradiation, sonochemical, photochemical and laser ablation. However the Turkevich citrate reduction method (reported in 1951), is still one of the most applied procedures, in which, sodium citrate reduces [AuCl4]- in hot aqueous solution to give colloids of 15-20 nm. In this method the size distribution of nanoparticles can be controlled by adjusting ration of gold salt to reducing agent, the temperature, and the order of addition of the reagents. Transmission electron microscopy (TEM) is the most common technique for characterization in term of average gold nanoparticles size and size distribution. Another very useful technique is UV-vis spectroscopy, which allows estimation of gold nanoparticles size, concentration, and aggregation level. Moreover, UV−vis spectrophotometers are present in most laboratories, the analysis does not alter the sample, and the registration of the spectrum requires short

times. The extinction spectra of gold particles recorded by this technique can be analyzed using the Mie theory.

In this study, gold nanoparticles were prepared by NaBH4 reduction method. Briefly, 100 ml of HAuCl4 aqueous solution (0.25 mM) were taken in 250 ml flask with magnetic stirring at 750 rpm and 0.1 M reducing agent (NaBH4) solution was added drop by drop with continuous stirring. The color of HAuCl4 solution changed from pale yellow to dark red over several minutes. Stirring process was continued for another 10 minutes for complete homogenization. After preparation of the particles, the dispersions were centrifuge at 14000 rpm for 15 min and the collected particles were redispersed in deionized water before any characterization. Several formulations were prepared by changing the concentration of reducing agent only, while all other parameters were kept constant. The average size of gold nanoparticles in various formulations were determined via different techniques such as dynamic light scattering, transmission electron microscopy, UV spectrum using standard curve and particles size calculated by using Mie theory and UV-vis spectrum of gold dispersion. Additionally, the polydispersity index was calculated from TEM images and effects of reducing agent concentrations were reported.

It was found that concentration of reducing agent did not affect the particle size and size distribution of gold nanodispersion up to certain limit (6.9 mM), however, when NaBH4 was used in excess, the particle size was increased with relatively broad size distribution. The NaBH4 concentration had slight effect on particle morphology too, and TEM images showed that by increasing reducing agent the practice color become darker and also probability of aggregations increases due to excessive reduction of gold salt. As the gold nanoparticle size increases, the the absorption peak shifts to a longer wavelength and full dark color of gold nanoparticle dispersion is the indication of high aggregation tendency of particle. Moreover, when the particle size was analyzed by UV standard curve based technique using standard curve, the obtained results were in agreement with particle size measured by DLS in samples where narrow size distribution. The UV standard curve based technique works better for fully monodispersed preparations. The nanoparticle size was found to be smaller when measured by TEM as compared to hydrodynamic particle size determined by DLS technique.

Nanoparticle size was also determined by using Mie theory based approach, optical absorption spectra of each sample were fitted using Lorentz equation and the particle size was found between 8 nm and 19 nm and almost in good agreement with those deduced from standard UV curve and TEM analysis. Thus gold nanoparticles can be prepared by $NaBH_4$ reduction method. And the average gold nanoparticle size can be evaluated by methods based

on the fitting of their UV−vis spectra by the Mie model for spheres, DLS, TEM and UV-vis spectrophotometer.

Preparation of gold nanoparticles and determination of their particles size via different methods.

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Abstract

Gold nanoparticles have been used in various applications covering both electronics, biosensors, in vivo biomedical imaging and in vitro biomedical diagnosis. As a general requirement, gold nanoparticles should be prepared in large scale, easy to be functionalized by chemical compound of by specific ligands or biomolecules. In this study, gold nanoparticles were prepared by using different concentrations of reducing agent ($NaBH_4$) in various formulations and their effect on the particle size, size distribution and morphology was investigated. Moreover, special attention has been dedicated to comparison of particles size measured by various techniques, such as, light scattering, transmission electron microscopy, UV spectrum using standard curve and particles size calculated by using Mie theory and UV spectrum of gold nanoparticles dispersion. Particle size determined by various techniques can be correlated for monodispersed particles and excess of reducing agent leads to increase in the particle size.

Keywords: A. Metals; A. Nanostructures: A. optical materials; B. Optical properties; C. Transmission electron microscopy

1. Introduction

Over the last three decades, nanoparticles research has received an increasing interest. This is due to the unique size dependent properties of nanoparticles, which are often thought as a separate and intermediate state of matter between individual atoms and bulk material (Schmid, 2006). Metal nanostructures present a wide variety of remarkable physical and chemical properties, which can be modified by changing their size, morphology, composition, and various preparation parameters (Abdelhalim and M. Mady, 2012; Schmid and Corain, 2003). Gold nanostructure have attracted considerable attention for many years because of their extensive use in various applications such as catalysis, photonics, electronics, optoelectronics, diagnostic, delivery, chemical, biological and biomedical sensing, photothermal therapy, surface plasmon resonance and surface-enhanced raman scattering (SERS) detection (Basu et al., 2012; Doria et al., 2012; Iodice et al., 2016; Kamiar et al., 2013; Kaya, 2011; Long et al., 2009; Lu et al., 2012; Sardar et al., 2009). Gold nanoparticles (AuNPs) are considered as good candidate for labeling applications due to its ability of strong interaction with visible light. Upon interaction with light, the excitation of free electrons in gold atoms lead to a state of collective oscillation called surface plasmon resonance (SPR), which provide gold the ability to absorb and scatter visible light depending upon its size, shape and agglomeration state (Huang et al., 2006; Kumar et al., 2007). AuNPs can be targeted and accumulated at specific tissue of interest thus enable visualization of that area under study. They can be detected by several techniques including phase contrast optical microscopy, dark field microscopy, photothermal imaging (Lim et al., 2011; Roth, 1996) and confocal scanning optical microscopy (Li et al., 2009; Sokolov et al., 2003). AuNPs have been reported to lack the capability to induce adverse and acute toxicity, thus, they are considered biocompatible device for biomedical applications (Connor et al., 2005; Costa Lima and Reis, 2015; Shukla et al., 2005; Singh et al., 2015). These properties of nanoparticles result from the extremely high proportion of surface atoms, this factor is directly dependent on the size of the nanoparticle. Indeed, the possibility to control these properties by adjusting the size of the nanoparticle, has been the cause of extensive investigation. Contrary to bulk materials that have constant physical properties regardless of mass, nanoparticles offer unique opportunities to control the electrical, magnetic and optical properties by modifying their diameter.

Nanoparticles can be prepared from various materials by relatively simple methods. In recent years, several types of methods have been published and reviewed. Currently, there

are two kinds of approaches commonly used to prepare nanoparticles, the “top down approach”, which involves the constant division of bulk metals into nanoparticles and the “bottom-up approach”, which involves the building up of nanoparticles from the atomic level (metal ions) (Iqbal et al., 2015; Kaya, 2011; Schmid and Corain, 2003). Various techniques such as, chemical, electrochemical, irradiation, sonochemical, solvothermal, photochemical and laser ablation have been used to prepare nanoparticles from metal ions precursors in the presence or absence of a capping agent (Ahmad et al., 2013; Akhavan et al., 2010; Fattori et al., 2013; Long et al., 2009; Okitsu et al., 2005; Wender et al., 2011). Michael Faraday was the first to study the formation of colloidal gold particles from a scientific point of view and used phosphorus agent for the reduction of $[AuCl_4]^-$ ions (Faraday, 1857). During the last century, numerous easy to handle reducers were found, such as sodium borohydride, thiosulfate, or organic ones like, sodium citrate, ascorbic acid, alcohols (polyalcohol) and amines (Li et al., 2011; Lloret et al., 2013; Long et al., 2009; Paul H. Davis et al., 2008; Salcedo and Sevilla III, 2013; Tabrizi et al., 2009). The Turkevich method is still one of the most applied procedures, in which, sodium citrate reduces $[AuCl_4]^-$ in hot aqueous solution to give colloids of 15-20 nm (Turkevich et al., 1951). Citrate itself and its oxidation products can act as protecting agents, even if no other stabilizer is used. However one of the most popular methods for preparation of gold nanoparticles of various sizes comes from Brust et al. It uses $NaBH_4$ to reduce gold salts in the presence of alkanethiols to yield gold particles of 1-3 nm. And, by varying the thiol concentration, the particles sizes can be controlled between 2 and 5 nm (Brust et al., 1994).

Characterization methods for analysis and measurement of nanomaterials are essential in the development of nanotechnology; as the sizes, shapes, and structures of nanomaterials influence their physicochemical properties. The most common technique used for characterization of metallic nanoparticles is high-resolution transmission electron microscopy (HRTEM), which generates a photomicrograph of the core of the nanoparticles, providing information regarding the particle size, size distribution and polydispersity of the samples. UV-visible (optical) spectroscopy is used for analysis of the intensely colored colloidal dispersions having characteristic surface plasmon absorption (Abdelhalim and M. Mady, 2012; Ibraheem et al., 2014; Iqbal et al., 2014). In a given preparation of nanoparticles, there is usually a mixture of different size particles, which, have characteristic surface plasmon resonance peaks and thus their UV-visible spectra are usually significantly different, which may help in determining the nanoparticle size (Haiss et al., 2007).

The aim of this work is to characterize the prepared nanoparticles in terms of morphology, size and size distribution. Special attention was dedicated to comparison of particles size measured by light scattering, transmission electron microscopy, by UV using standard curve and the particles size was calculated using Mie theory and UV spectrum of gold dispersion.

2. Materials and methods

2.1. Materials

Gold (III) chloride hydrate (≥99.999%) was purchased from Sigma Aldrich, and sodium borohydride ($NaBH_4$), 98+%, was purchased from Acros Organics. Water was deionized using (Aquadem® from Veolia Water, France). Nitric acid (68%) and Hydrochloric Acid (35%) were obtained from BDH Prolabo-VWR International.

2.2. Preparation of gold nanoparticles

The preparation of gold nanoparticles was performed by $NaBH_4$ reduction method as described in literature (Selvakannan et al., 2003). Briefly, 10 mg of $HAuCl_4$ was dissolved in 100 ml of deionized water (≈ 0.25 mM), and shaken properly to mix the solution. And, 0.1 M solution of reducing agent ($NaBH_4$) was prepared by dissolving 1.891 g of $NaBH_4$ in 500 ml of deionized water. Then, 100 ml of $HAuCl_4$ (0.25 mM) were taken in 250 ml flask with magnetic stirring at 750 rpm (230 V, IKA® RET, Germany) and the reducing agent solution was added drop by drop with continuous stirring. The color of $HAuCl_4$ solution changed from pale yellow to dark red over several minutes. Stirring process was continued for another 10 minutes for complete homogenization. Since the $HAuCl_4$ is corrosive, a glass spatula was used to avoid the contact with metal. In the preparation of gold nanoparticles, cleaning of glassware is very crucial. Thus, all the glassware and stir magnetic bars were thoroughly cleaned in freshly prepared aqua regia (HCl/HNO_3 3:1, v/v) and then rinse with distilled water and dried, to avoid aggregation of residual gold particle and to avoid unwanted nucleation during synthesis. After preparation of the particles, the dispersions were centrifuge at 14000 rpm for 15 min and the collected particles were redispersed in deionized water before any characterization. All the gold nanoparticles batches were store in the dark to minimize the photo induced oxidation.

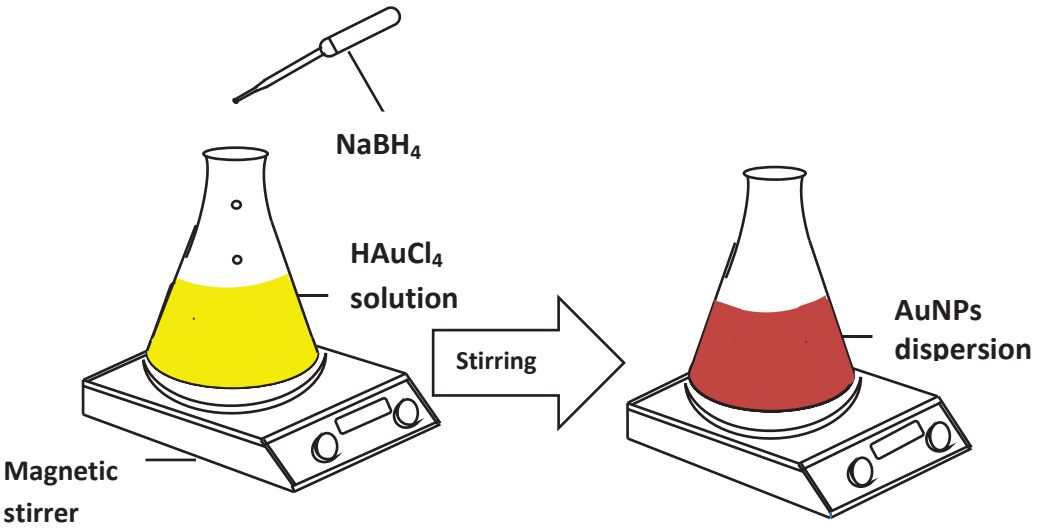

Fig. 1. Schematic illustration of gold nanoparticles preparation process using $NaBH_4$ as reducing agent

2.3. Physicochemical characterization of nanoparticles.

2.3.1. Hydrodynamic particle size measurement

The hydrodynamic particles size (Dh) of the colloidal dispersions was determined by dynamic light scattering (DLS) using zetasizer from Malvern (England) at room temperature (25 C°). The mean hydrodynamic diameter is calculated by using the Stokes–Einstein's equation:

$$Dh = \frac{kT}{3\pi\eta D} \quad (1)$$

Where, k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium, and D is the diffusion coefficient. Each sample was prepared by adding 2 ml of the prepared nanoparticles dispersion in quartz cell and then the cell was placed in zetasizer for analysis. Mean particle size was determined at a scattering angle of 90° for all the samples. The particle size was determined in triplicate for all samples, and then averaged.

2.3.2. Transmission electron microscopy morphology and particles size analysis

Transmission electron microscopy (TEM) was performed with a Philips CM120 microscope at the "Centre Technologique des Microstructures" (CTμ) at the University of Lyon (Villeurbanne, France). A small drop of suspension was deposited of a microscope grid (copper support covered with carbon) and slowly dried in open air. The dry samples were observed by TEM under 120 kV acceleration voltages. The average gold nanoparticles diameter and polydispersity index (PDI) were calculated for each sample by averaging 200

particles from the TEM images using ImageJ software (image processing program developed at the National Institutes of Health)

2.3.3. Size determination using reported standard curve

The absorbance of gold nanoparticles dispersions was examined using spectrophotometer (UV-1800 Shimadzu, Japan). The washed dispersions were redisposed in deionized water and the absorbance was recorded from 190 nm to 750 nm as a function of wavelength using quartz cell with a path length of 1 cm. Two major information were extracted from the obtained spectrum i.e the maximum wavelength (λ*max)* and full width at half maximum (*FWHM*). The obtained λ*max* were used for particle size estimation by using standard curve (Ghosh et al., 2004) and the deduced λ*max and FWHM*used for the calculation of particles using Mie theory. Various dispersions of gold nanoparticles were prepared and then analyzed by a double beam UV-vis spectrophotometer (Shimadzu UV-1800) in the range of 190 nm to 750 nm. From the obtained spectra, maximum wavelength were extracted and used to estimate the particle size from the reported standard curve from *Ghosh et al.*(Ghosh et al., 2004) to access to the gold nanoparticle's size.

Table 1

Data from Ghosh et al.(Ghosh et al., 2004) in which particle size and maximum wavelengths absorption of gold nanoparticles are reported as a function of trisodium citrate concentration.

Run	$HAuCl_4$ solution (10mM, mL)	Trisodium citrate solution (1%, mL)	Color	λmax	Average diameter (nm)	
					Reported	Observed
A	1.25	2.000	Dark red	518	-	8.00
B	1.25	1.600	Red	519	-	10.0
C	1.25	1.300	Red	520	-	13.0
D	1.25	1.000	Red	522	16.0	16.0
E	1.25	0.875	Red	526	-	20.0
F	1.25	0.750	Red	528	24.5	25.0
G	1.25	0.625	Pinkish red	529	-	32.0
H	1.25	0.500	Pink	532	41.0	41.0
I	1.25	0.400	Pink	534	-	55.0
J	1.25	0.300	Orange	545	71.5	73.0

In order to determine the particles size of our samples, the data reported in Table 1 is used to establish the standard curve reported in Fig. 2.

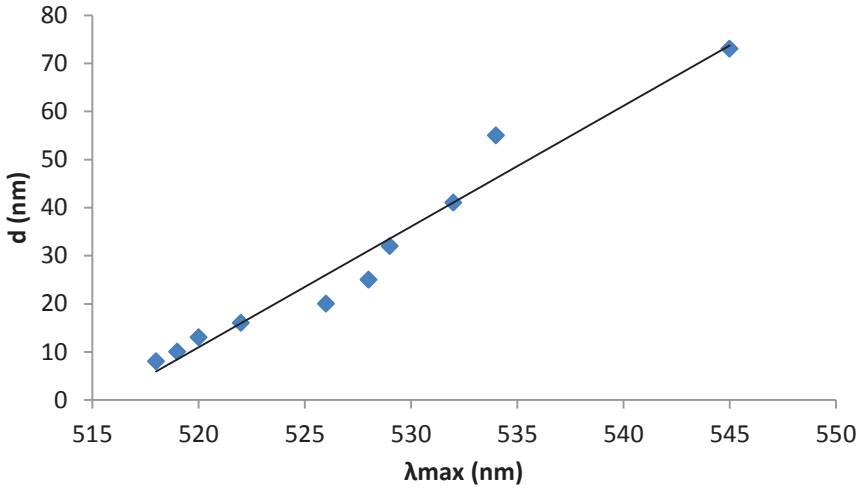

Fig. 2. Standard curve of particle size versus wavelength (nm) deduced from Table 1.

The reported data in Fig. 1 is fitted by the following linear equation:

d (nm) = 2.511 λmax (nm) - 1294.8. **(2)**

This equation gives the relation between nanoparticles size and maximum wavelength that will be used to estimate the particle size of the gold prepared particles.

2.3.4. Size determination using UV-vis Spectroscopy and Mie theory

The size, concentration, and, in some cases, aggregation level of AuNP are key points for nanoparticles applications because they determine chemical, optical , electrical and biological properties (Amendola and Meneghetti, 2009; Schmid and Corain, 2003). The estimation of the average size of gold nanoparticles based on the fitting of their UV-vis spectra by the Mie model for sphere was used and explored in this study (Amendola and Meneghetti, 2009; Haiss et al., 2007). In fact, the average diameter d of various noble metal (Ag, Au, Pt) can be estimated from electromagnetic theory of Mie (Akbari, 2011; Amendola and Meneghetti, 2009; Desai et al., 2012; Prikhodko et al., 2014) using the half-width of resonance optical absorption peak and characteristic wave length of plasmon resonance "λp".

$$d = \frac{vf\,\lambda p^2}{\pi c \Delta\lambda} \qquad (3)$$

Where “vf” is the electron velocity corresponding to the fermi energy of the metal, “c” is the velocity of light, $\Delta\lambda$ is the full width at half maximum of absorption band, λp is the characteristic wave length at which SPR occurs (Manikandan et al., 2003) . In order to find all these parameters; (full width at half maximum -FWHM, SPR position, absorbance intensity) spectra were fitted to Lorentzians. We have employed “ORIGIN 8.0” software. Utilization of the Mie model consists in an estimation of the average radius by fitting the 300-800 nm spectral regions.

3. Results and discussion

As above mentioned, various gold nanoparticles samples are prepared using the same recipe but not the same amount of reducing agent in order to know the effect of reducing agent concentration on particle size, size distribution and morphology. The obtained gold dispersions were characterized in term of hydrodynamic particles size, size distribution using light scattering. The morphology, the particle size and polydispersity were calculated from TEM image. The intrinsic photophysical property of the prepared gold nanoparticles was examined by spectrophotometry and the particles size was then examined using reported standard curve or by using Mie theory. The obtained results are reported in Table 2 as a function of used method, approach and recipe.

Table 2

Composition of various formulations for preparation of gold nanoparticles. The concentration of HAuCl4 was fixed (0.25mM, 100 ml) for all formulations; only the concentration of reducing agent (NaBH4) agent was modified by changing volume of NaBH4 (0.1M). The nanoparticles sizes determined by using different techniques are tabulated.

Formulation code	Concentration(mM) of $HAuCl_4$	Concentration(mM) of $NaBH_4$	λmax (nm)	PDI	Particle size (nm) calculation versus used methods			
					DLS	UV	TEM	Theore-tical
F1	0.25	2.9	520	1.034	15.4	10.9	11.4	13.10
F2	0.25	3.8	525	1.059	18.8	23.5	13.7	8.42
F3	0.25	4.7	520	1.054	17.0	10.9	14.6	9.76
F4	0.25	6.5	523	1.035	19.4	18.5	10.1	9.70
F5	0.25	7.4	533	1.044	18.2	43.6	16.2	14.14
F6	0.25	6.9	523	1.049	17.7	18.5	15.6	8.10
F7	0.25	11.5	536.5	1.104	30.3	52.4	22.3	19.22

The physicochemical aspect of colloidal system such as, particle size and zeta potential, are known to influence the physical stability of colloids, release rate and their interaction with cells and biological environment. The particles were characterized on the basis of morphology, particle size and size distribution.

3.1. Transmission Electron Microscopy morphology analysis

Transmission electronic microscopy (TEM) was performed in order to visualize the morphology of nanoparticles prepared by $NaBH_4$ reduction of chloroauric acid solution. Particles were evaluated on the basis of shape, size, size distribution, and presence of interparticles bridging and aggregation. Under TEM observations, the nanoparticles produced had spherical shapes (Fig. 4), with an average particle size smaller than 30 nm. The average particle sizes were also confirmed by light scattering analysis (Table 2). TEM Images showed slight aggregation between some particles (Fig. 4(F7a) and (F7b)), this can be attributed to

excessive reduction of gold salt solution; since comparatively high concentration of reducing agent was used in this formulation. However, at low reducing agent concentrations the particles were fairly detached and homogeneous (Fig. 4(F6a) and (F6b)).

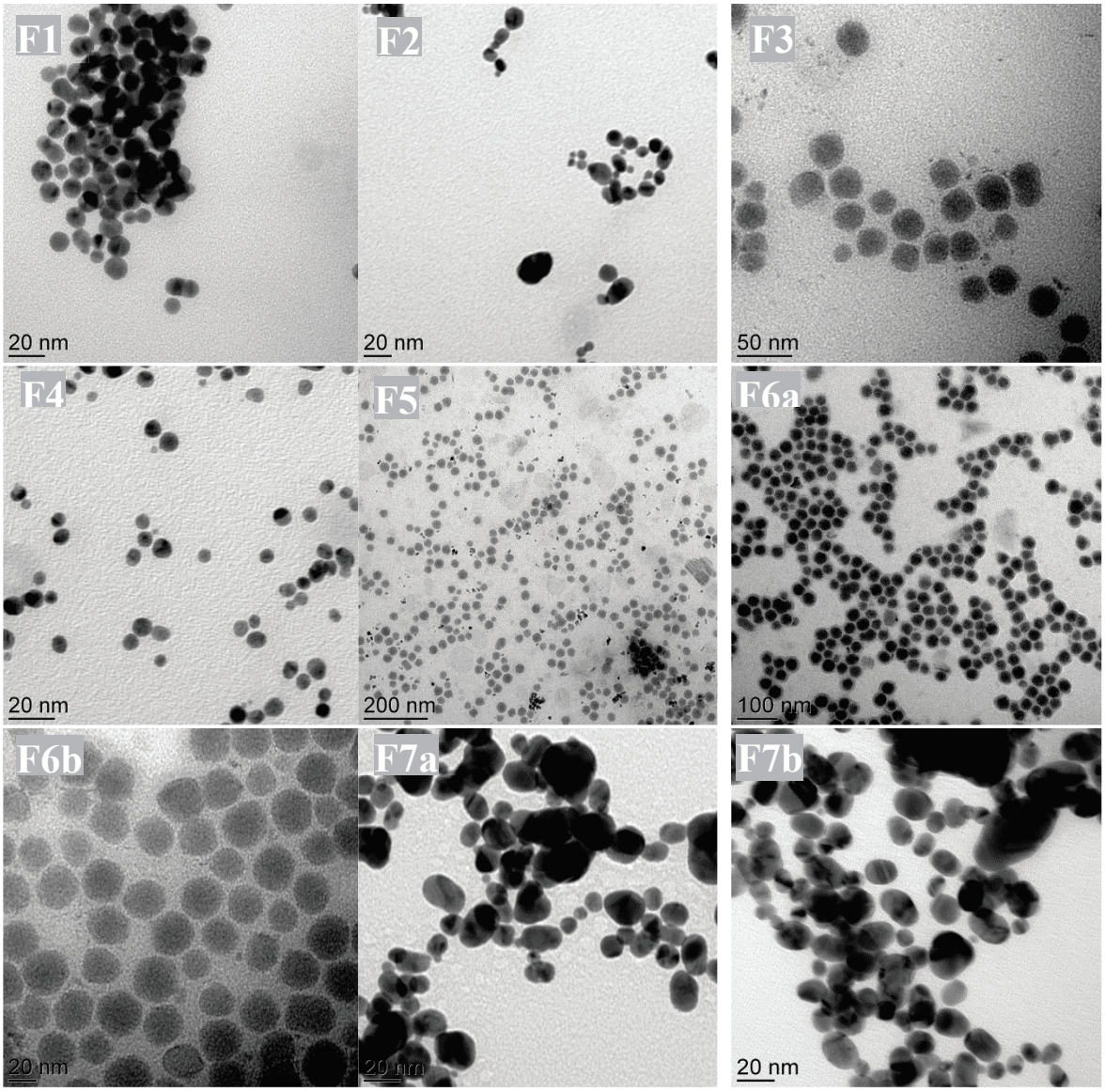

Fig. 3. TEM micrograph of formulations prepared with different concentration of reducing agent (F1) 2.9 mM, (F2) 3.8 mM, (F3) 4.7 mM, (F4) 6.5 mM, (F5) 7.4 mM, (F6a,b) 6.9 mM, and (F7a,b) 11.5 mM. Scale bars represent 50 nm in F2 and F3, 100 nm in F6a and 20 nm in all of the rest.

3.2. Particle size analysis via various methods

3.2.1. Light scattering and TEM Particles size and size distribution analysis

Different dispersions of gold nanoparticles were prepared using various concentrations of reducing agent, while the concentrations of $HAuCl_4$ were kept constant

throughout all formulations. Seven samples (F1, F2, F3, F4, F5, F6, and F7) were prepared by using freshly prepared reducing agent solution ($NaBH_4$). From the obtained results, it was found that, by increasing the concentration of reducing agent from 2.9 mM (F1) to 7.4 mM (F5), there was no significant effect on particle size in all formulations and the average particle size was found between 15.4 nm and 19.4 nm when measured by using DLS technique (Table 2). However, at high concentration of reducing agent (11.5 mM) the particle was increased to 30.3 nm. Similar trend of slight increase in particles size was also found, when it was calculated from TEM images. The average particle size calculated from TEM, was smaller compare to hydrodynamic particle size as shown in Table 2, which is logical because, usually hydrodynamic particle size is larger than TEM particle size. The obtained results showed that particle size increases with an increase in reducing agent concentration. This may be due to aggregation and over reduction of gold salt in the presence of excess of reducing agent. Though, Ghosh et al reported a decrease in particle size with an increase in reducing agent amount, however, they prepared NP via Frens' method by using sodium citrate as reducing agent instead of NaBH4 with adsorbed pyrene on their surface (Ghosh et al., 2004). The polydispersity index (PDI) was calculated for each sample from the TEM images using ImageJ software and PDI was found between 1.034 and 1.104 (Table 2). The formulation F7 showed relatively high PDI due to slight attachments of particles.

The particle size distribution, which reflects the polydispersity of colloidal system, was also examined. It was observed that, with an increasing in $NaBH_4$ concentration, there was slight increase is size distribution graph as shown in Fig. 4(C). This is also evident from TEM images, which shows some particle aggregation in Fig. 4(F7a) and (F7b). This may be due to high degree of gold salt reduction leading to slight aggregation, as no stabilizing agent was used in all formulations. Increase in particle size and size distribution by increasing reducing agent concentration has been already reported (Tabrizi et al., 2009). The high surface energy of AuNPs makes them very reactive, which mostly leads to aggregation of particles without protection of their surfaces (Guo and Wang, 2007). However, at low reducing agent concentration (F6) the size distribution plot was narrow (Fig. 4(A) and (B)) with homogenous particle size.

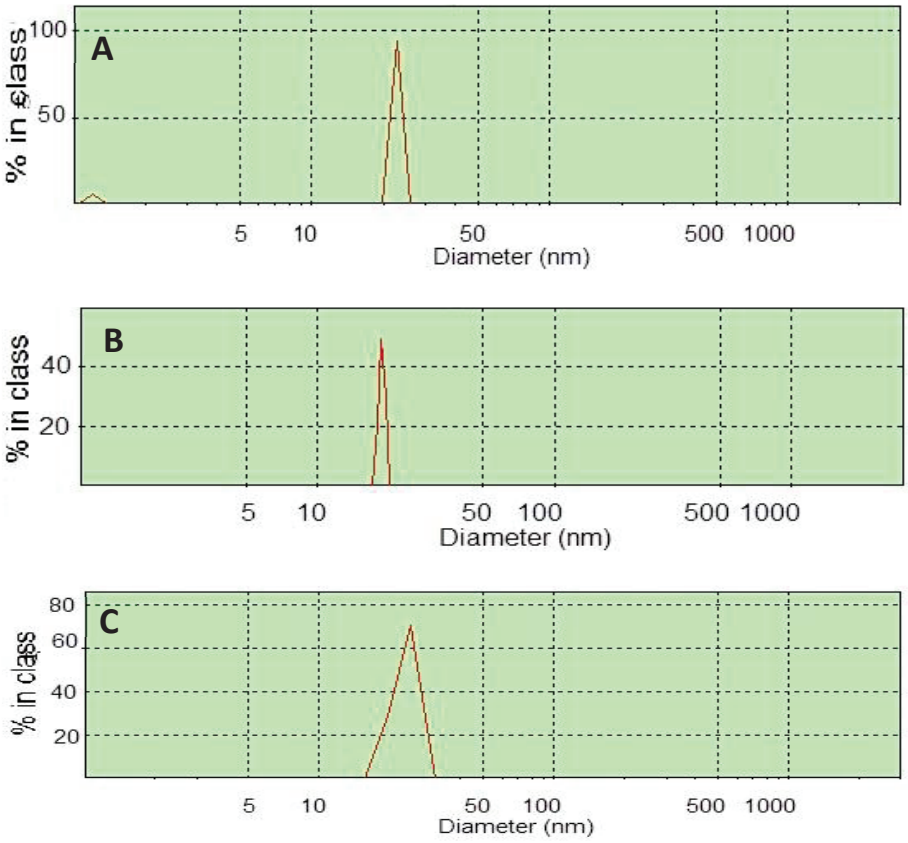

Fig. 4. Particle size distribution of various formulations prepared with different concentration of reducing agent (A, B and C represent sample F4, F6 and F7 respectively).

3.2.2. Particles size analysis by UV standard curve based method

For determination of particle size of our samples, we used the Eq. 2, which was derived from the standard curve (Fig. 2) based on the data taken from Table 1 (Ghosh et al., 2004). For this purpose, first, the wavelength of maximum absorbance (λmax) for each sample of prepared nanoparticle dispersion was determined by using UV spectrophotometer (Table 2). Subsequently, the particle size was calculated for each formulation by putting their respective λmax values in Eq. 2, which relates the λmax with particle size (diameter).

Each formulation showed specific maximum wavelength (λmax), which reflect their photochemical characteristics. The λmax of different formulations was found between 520 nm and 536.5 nm (Fig. 5), and the particles size calculated were in the range of 10.97 nm to 52.40 nm (Table 2). A tendency of an increase in absorbance was found as the particle size increase, which were in agreement to those previously reported (Iwamoto et al., 2005; Njoki et al., 2007). The absorbance increase is due to the progressive increase in particle size; larger

particles have larger molar extinction coefficient values(Kuo et al., 2004; Link and El-Sayed, 1999; Verma et al., 2014). Moreover, the particle size obtained from this calculation was in agreement with particle size determined by DLS (Table 2) in case of samples F2, F4 and F6, whereas slight deviations are shown in samples F5 and F7. The particle size calculated by this technique was large than DLS results, this may be due to partial aggregation of particles, especially in sample F7, which can also be seen from TEM images (Fig. 3(F7)). This equation (Eq. 2) can be ideal for completely monodispersed preparations and can be used to obtain information regarding nanoparticles polydispersity.

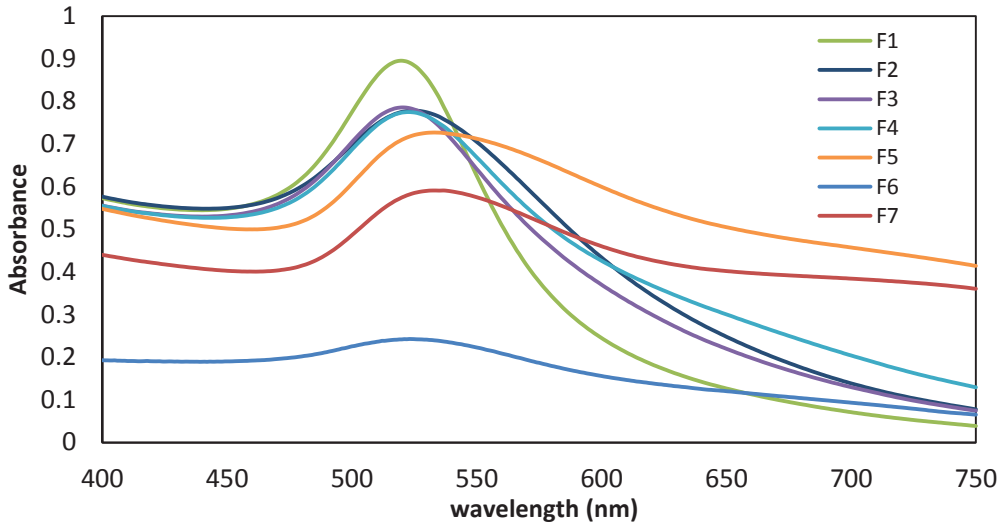

Fig. 5. Absorption spectra of gold nanoparticles dispersion prepared with different concentration of reducing agent.

3.2.3. Size determination using UV-vis Spectroscopy and Mie theory

AuNPs show strong plasmon resonance absorption that is dependent on the particle size, shape and agglomeration. For almost spherical gold nanoparticles, the plasmon band maximum is generally between 520 and 530 nm. (Jana et al., 2001a, 2001b; Shimizu et al., 2003). In order to determine the particle size, the obtained data of optical absorption spectra of each sample were fitted using Lorentz equation as below illustrated in Fig. 6 (for sample F6) in which optical absorption spectra was presented and mathematically fitted.

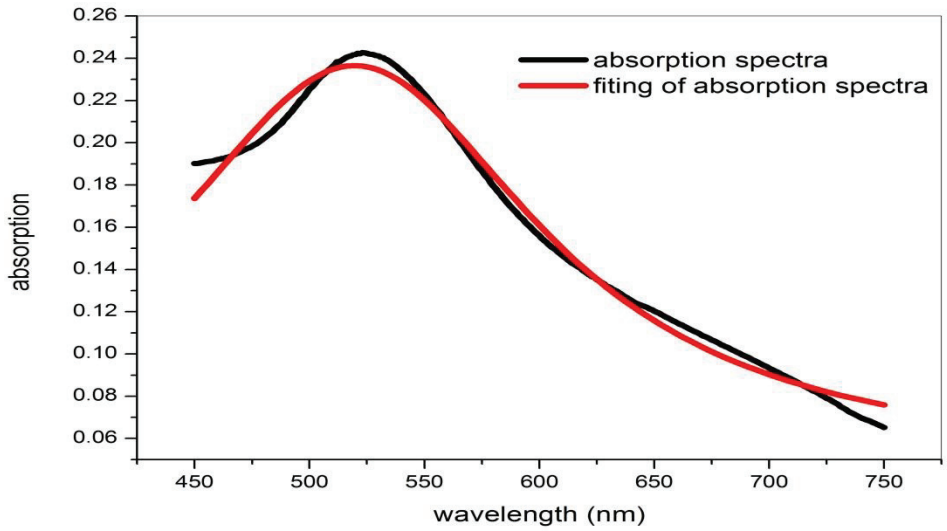

Fig. 6. Optical absorption spectra of the prepared gold nanoparticle (Lorentz fit of sample F6) best fit of the optical surface plasmon absorption spectra using Mie equation.

The results of particles size estimation deduced from the best fitting using Mie theory are listed in Table 2. The determined diameters are in between 8 nm to 19 nm and almost in good agreement with those deduced from standard UV curve and TEM analysis. This method is more accurate and more establish theory compared to UV standard-based method. This Mie theory based approach has been already examined and totally approved as already reported (Haiss et al., 2007). The reported results showed that for the particle size larger than 25 nm, both the theoretical and experimental peak positions precisely fit better compared to particle size smaller than 25 nm. This may be attributed to the proclaimed increase of the ratio of the surface atoms to bulk atoms for particle diameter smaller than 20 nm. Amendola et al. (Amendola and Meneghetti, 2009) show that the size of free or functionalized gold nanoparticles in water and other solvents, with diameters in between 4 and 25 nm, can be measured with an accuracy of about 6%. The literature value for Plasmon bands is usually in between 520 and 530 nm for spherical gold nanoparticles (Schmid and Corain, 2003). The maximum and the bandwidth of the plasmon band are both strongly dependent on the size and interactions with the surrounding medium. One can see that the SPR width increases for decreasing sizes in the 4-28 nm intervals.

4. Conclusion

In this study, the gold nanoparticles were fabricated that can be used for biomedical applications as imaging contrast agent both in vitro and in vivo. The effect of reducing agent concentration on the particle size, size distribution and morphology was investigated and also

the particle size was determined via different techniques such as, DLS, TEM, UV-vis spectrophotometry and Mie theory. It was found that concentration of reducing agent did not affect the particle size and size distribution of gold nanodispersion up to specific concentration (F6), however, when $NaBH_4$ was used in excess, the particle size was increased with relatively broad size distribution at high concentration of reducing agent. The reducing agent concentration had slight effect on particle morphology too, and TEM images showed that by increasing reducing agent the practice color become darker with distinct boundaries of particles and also probability of aggregations increases due to excessive reduction of gold salt. Moreover, when the particle size was analyzed by UV standard curve based technique using standard curve, the obtained results were in agreement with particle size measured by DLS in samples where low concentration of reducing agent was used, however, at high concentration of $NaBH_4$ the particle size calculated was larger than those of DLS technique because of slight particles aggregation in the sample as already reported in literature, that UV standard curve based technique works better for fully monodispersed preparations. The nanoparticle size was also determined by using TEM images, and the average particle size was found to be smaller as compared to hydrodynamic particle size determined by DLS technique. Nanoparticle size was also determined by using Mie theory based approach, optical absorption spectra of each sample were fitted using Lorentz equation and the particle size was found between 8 nm and 19 nm and almost in good agreement with those deduced from standard UV curve and TEM analysis.

From this study, it was concluded that the gold nanoparticles can be prepared successfully by $NaBH_4$ reduction of $HAuCl_4$, and their particle size can be verified through different methods. Furthermore, these particles can be used in biomedical imaging techniques as contrast agent such as MRI to visualize different tissues both for in vivo and in vitro applications and AuNPs can be surface functionalized for other potential applications in several field. These particles can be co-encapsulated with anticancer active agent for theranostic purpose.

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IV. Discussion, conclusion and future perspectives

Discussion and conclusion

Nanotechnology has great potential for early detection, accurate diagnosis, and personalized treatment of many fetal diseases. Its application in biomedicine has been extensively studied over the last decade. Due to their specific size and shape, nanoparticles offer multifunctional capability by overcoming the numerous biological, biophysical, and biomedical barriers, which may revolutionize diagnosis and treatment of several diseases. It can offer unprecedented interactions with various biomolecules both on the surface of and inside the cells, and can offer platforms for efficient and targeted delivery of drugs and imaging agents for in vitro and ex vivo applications. The first step is the selection of appropriate technique for preparation of nanoparticle with required characteristics for intended application. Here, modified double emulsion solvent evaporation method was used for preparation of polymeric particle. This technique is appropriate for the encapsulation of both hydrophilic drugs and hydrophobic drugs. Moreover, it allows flexibility in particle size by adjusting various process parameters, the process is independent of special laboratory equipment, cost effective and preferable for low scale production. Conversely, single emulsion process allow for the encapsulation of hydrophobic molecules into a multitude of particulate materials, yet their application in hydrophilic compounds encapsulation is limited due to uncontrolled leakage of entrapped compounds during particles preparation. Polycaprolactone was selected as polymer due to its desirable properties such as biocompatibility, biodegradability and non-toxic nature. It has low glass transition temperature and melting point (60 °C), and the polymer metabolites are removed from the body by innate metabolic process and do not produce acidic environment as in case of PLA and PLGA. Its compatibility with variety of drugs, and its slow degradation rate to release drug for prolonged period of time makes it a suitable candidate for controlled drug delivery systems. Moreover, it allows the modification of its physicochemical and mechanical properties by copolymerization, which intern affect all other properties of polycaprolactone including solubility, ionic property and degradation pattern. Dichloromethane was chosen as solvent due to low boiling point (39.6 °C), immiscibility with water and its ability to dissolve polycaprolactone properly.

As drug carriers, the large surface area of nanoparticles can enhance drug dissolution and they are capable to improve controlled release compared to micron-sized drug carriers. Though, their tendencies of aggregate are the potential problems to be overcome, therefore an appropriate

stabilizer may be needed to avoid aggregations. Poly vinyl alcohol (PVA) is a common stabilizer that has been used as an excipient in a wide range of pharmaceutical formulations. Here, PVA was used as stabilizer in the second step of emulsification process in order to facilitate homogenization and to prevent aggregation. It was used in different concentration in order to investigate the optimum concentration of PVA, and 0.5% concentration of PVA showed desirable results. Since, the physicochemical properties of the polymeric particle prepared via modified double emulsion are affected by the process parameters therefore various parameters were thoroughly investigated and the optimized parameter were used for the encapsulation of contrast agents and active drugs. Polycaprolactone particle were prepared via double emulsion process by using two homogenization technique i.e mechanical (ultra-turrax) homogenization and sonication homogenization. In utra-turrax process, the effects of stirring time and stirring speed in both, the first step and the second step of emulsification were investigated. Moreover, the effects of PCL amount, PVA concentration and outer aqueous phase volume, on particle size, size distribution and zeta potential were investigated. The size of obtained particles through this technique was in micron range. Since, our goal was to obtain sub-micron particle therefore, we adopted sonication hominization for preparation of double emulsion. And, the effects of sonication process parameters were investigated in order to point out the relationship between the used conditions and the colloidal properties of obtained dispersion. The studied parameters were included, ultrasound exposure time in the first step and the second step of emulsification, sonication amplitude, polymer amount, PVA concentration and outer aqueous phase volume ratio. The particles obtained via double emulsion sonication homogenization were in submicron range (300-500 nm). Ultrasound exposure time is a key parameter affecting ultrasonic emulsification process. In both, the first and the second step of emulsification, 2, 4, 6, 8, and 10 min sonication time was used. In the first step, sonication time had no significant effect on the particle size and morphology while in the second step, the particle size gradually decreased with the increase in ultrasound exposure time. The ultrasound amplitudes used in this study were 50%, 60%, 70%, 80%, and 90%. Initially, when 50% of amplitude was used, no homogenization was observed and both, the oily phase and the aqueous phase of double emission were distinctly visible. This was probably due to insufficient energy transmitted to the emulsion system to induce cavitation. Once cavitation threshold was reached, by increasing amplitude to 60% and above, then a proper homogenization of the system was achieved. There was tendency of decrease in

particle size with the increase in amplitude and the smallest particles were obtained at 90 % sonication amplitude. Various formulations were prepared with different amount of polymer in order to study its effect and we found that by increasing the polymer amount the particle size also increases and the particle with good morphology were obtained at 3 g of PLC. This increase in particle size with increase in PCL solid content can be attributed to increased viscosity of the primary emulsion, which leads to less particle size reduction during second step of emulsification. The addition of suitable stabilizer plays an important role in colloidal dispersions. The nature and concentration of stabilizer affect the colloidal stability of the prepared dispersion. In this study, concentration effect of PVA in the outer aqueous phase over average hydrodynamic particle size and morphology of particles were evaluated. When PVA concentration was decreased beyond 0.2% there was drastic increase in particle size, this may be due to coalescence of droplets, since, this amount of PVA was insufficient to cover properly the nanoparticles surfaces and no homogenization was achieved in the absence of PVA. There was no significant effect of PVA concentration above 0.2 %, and optimal morphology of particles was found at 0.5% of PVA concentration.

Generally, imaging is performed for diagnosis of a disease state before therapy of several diseases like cancer by using proper imaging technique. Several imaging technologies (Magnetic resonance, optical etc.) depend on contrast agent, highlighting the differences between tissues thus, allows efficient visualization of the tissues of interest. Contrast agents are encapsulated in order to enhance their stability and to deliver high payload of contrast agents to target specifically. In the light of these optimized parameters, the fluorescent contrast agent (FluoSpheres®) was loaded into polycaprolactone particles in different concentration. The contrast agent-containing submicron particle was characterized in term of average particle size, morphology and encapsulation efficiency. Moreover, contrast agent distribution in the PCL matrix was determined by confocal microscopy. The incorporation of contrast agent in different concentrations did not affect the physicochemical properties of PCL particles and the average size of loaded particles was found to be in the submicron range. However, the particles loaded with high fluorescent argent concentration showed high fluorescence intensity when visualized by confocal microscopy, which show their proper encapsulation into PCL matrix. The average size of loaded particle were about 322-375 nm and it was not affected by variation in loaded fluorescent agent amount. The encapsulation efficiency was found to be high enough i.e from

84% to 91% in different formulations. The skin penetration study was performed and red fluorescence allowed easy identification of loaded particles on the surface of skin and hair follicles. PLC particles remained on the surface of both stratum corneum and under passive diffusion conditions, no penetration of particles was found. Since, impermeability of the stratum corneum is the main barrier to cutaneous molecule absorption. Another important issue is the mechanical stress applied to the skin when investigating the penetration behavior. Therefore the application of a mechanical stimulation to the skin may be used to enhance its permeability. The zeta potential was found near to zero this may be due to non-charged nature of the polycaprolactone polymer.

Another contrast agent frequently used for imaging in biomedical applications targeting theranostic is gold nanoparticles. They play an important role in in vitro assays, ex vivo and in vivo imaging, and drug delivery. A multifunctional platform based on gold nanoparticles have several capability such as, targeting , multimodal imaging, delivery of therapeutic moieties, thus, holds the promise for a “magic gold bullet” against cancer and other fetal disease. First, gold nanoparticles were prepared by NaBH4 reduction technique. 100 ml of HAuCl4 (0.25 mM) solution in deionized water was reduced by adding NaBH4 dropwise and stirred via magnetic stirring at 750 rpm. The relationship of various NaBH4 concentrations in different formulations, with respect to the nanoparticle size and morphology was evaluated. The average sizes of gold nanoparticle were about 20 nm at low concentration of NaBH4 (3-7 mM) however at 11.5 mM of NaBH4 the particle size was increased to 30 nm and the color of dispersion was changed from dark red to dark, which show that over reduction of gold salt leads to aggregation of particle. The gold nanoparticle size was measured by using various approaches such as, dynamic light scattering, transmission electron microscopy, UV spectrophotometry using standard curve and calculated particles size using Mie theory and UV spectrum of gold dispersion and the obtained results were compared with each other.

After well characterization, the prepared gold nanoparticles were then encapsulated by polycaprolactone matrix, to be used as MRI or optical contrast agent. And in the next step, the PCL submicron particles were loaded with gold nanoparticles as contrast agent, a hydrophilic drug (Nefopam) and a lipophilic drug (benzyl benzoate) simultaneous to fabricate a multifunctional particle. The encapsulation efficiency of benzyl benzoate was almost 100 percent

as it is a lipophilic drug thus, having no affinity of leakage from polymeric matrix to outer aqueous phase.

Perspectives:

In future, further study will be conducted regarding pharmacokinetic and pharmacodynamics of the contrast agents and the drug encapsulated via double emulsion process. For skin penetration study of contrast agent loaded particle, we will evaluate the impact of the removal of the stratum corneum and the application of a mechanical stimulation. The obtained results provide valuable information regarding particles preparation and encapsulation via modified double emulsion process and their possible application in cancer therapy. In light of this study, various drugs and imaging agent will be encapsulated for different diseases using theranostic approach. It will be applied to other route of administrations. Since, multifunctionality is the key feature of theranostic agents. Therefore, targeting ligands, imaging labels, multiple therapeutic drugs, and other functionalities can all be integrated to allow for targeted molecular imaging and therapy of various diseases via theranostic approach. The successful achievement of all these goals will be helpful in timely and better management of various fetal diseases.

Préparation de particules submicroniques pour applications théranostiques: imagerie et thérapie

Résumé

L'objectif de cette étude était de préparer et de caractériser les particules submicroniques multifonctionnelles utilisables simultanément pour le diagnostic et le traitement de plusieurs maladies mortelles telles que le cancer. Pour ce faire, une étude systématique a été réalisée afin de comprendre les mécanismes impliqués et d'optimiser les paramètres du procédé de double émulsion-évaporation de solvant pour la préparation de ces particules. Pour l'imagerie in vitro, des nanoparticules polymériques fluorescentes (FluoSpheres®) ont été encapsulées dans une matrice polycaprolactone dégradable en utilisant le procédé de l'émulsion double-évaporation de solvant. Pour l'imagerie in-vivo, des nanoparticules d'or colloïdal ont été préparées et encapsulées via le même procédé et parfaitement caractérisées. Enfin, pour application theranostic, les nanoparticules d'or (comme agent de contraste) et un actif moléculaire (hydrophile Nefopam et hydrophobe benzoate de benzyle) ont été encapsulés simultanément dans des particules de polycaprolactone. Ces particules multifonctionnelles ont été caractérisées et évaluées in vitro comme model de pénétration cutané

Mots clés: Encapsulation, les particules submicroniques, Imaging, Théranostic, Double émulsion, les nanoparticules d'or, Agent de contraste.

Preparation of submicron particles for theranostic applications: imaging and therapy

Summary

The objective of this study was to prepare and characterize multifunctional submicron particles that can be used for diagnosis and therapy of several fatal diseases including cancer (i.e theranostic). For this purpose, a systematic study was performed in order to optimize the process parameters for preparation of polymeric particle that can be used as a platform for effective delivery of drugs and imaging labels. The imaging agent (FluoSpheres®) was encapsulated via double emulsion solvent evaporation technique to be used fluorescent contrast agent and their in vitro evaluation was performed. Then, gold nanoparticles were prepared by using NaBH4 reduction method, characterized and encapsulated by polycaprolactone polymer for in vitro applications. Finally, the gold nanoparticle were loaded into polycaprolactone particle along with a hydrophilic drug (Nefopam) and a hydrophobic drug (benzyl benzoate) simultaneously. The prepared particles were then characterized physicochemically and in vitro skin penetration study was performed.

Keywords: Encapsulation, Submicron particles, Imaging, Theranostic, Double emulsion, Gold nanoparticles, Contrast agent.